Nicotiana attenuata protein kinases mediate plant growth and resistance to insect herbivores

Dissertation

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von Diplom Biologin Maria Heinrich

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Gutachter

1. Prof. Ian T. Baldwin, Max Planck Institute für chemische Ökologie, Jena

2. Prof. Dr. Ralf Oelmüller, Institut für Pflanzenphysiologie, FSU Jena

3. Prof. Dr. Markus Teige, Universität Wien

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Chapter 1: General Introduction

Plants evolved sophisticated strategies to defend themselves against biotic and abiotic stresses. Highly complex signaling networks control these defense responses (Howe & Browse, 2008; Wu & Baldwin, 2010). Within these networks, the mitogen-activated protein kinase (MAPK) cascades play essential roles (Ichimura *et al.*, 2000; Asai *et al.*, 2002; Teige *et al.*, 2004; Meszaros *et al.*, 2006; Andreasson & Ellis, 2010).

MAPKs are well conserved in eukaryotes and they play a key role in transducing extracellular stimuli to intracellular responses (Romeis, 2001; Zhang & Liu, 2001). Typically, a MAPK cascade consists of a three-kinase module. MAPKs form the terminal components of these cascades, and are activated by MAPK kinases (MAPKKs) via dual phosphorylation of conserved threonine and tyrosine residues. MAPKKs are themselves activated through phosphorylation of conserved serine and/or threonine residues (MAPK Group *et al.*, 2002). The genome of the model plant *Arabidopsis thaliana* encodes 20 MAPKs, 10 MAPKKs and more than 60 MAPKKKs (Andreasson & Ellis, 2010). This mismatch between the numbers of MAPKKs and MAPKs suggests that individual MAPKKs must have the capacity to activate more than one MAPK (Andreasson & Ellis, 2010). Several recent studies support this scenario. The Arabidopsis MAPKs, MPK3 and MPK6, have been shown to be regulated after different stimuli, and MPK3 and MPk6 are phosphorylated by different MAPKKs. They seem to be regulated by the MKK9 (a MAPKK) in ethylene signaling (Yoo *et al.*, 2008). Additionally, both MAPKs are regulated by the MAPKKs, MKK4/MKK5, to confer resistance to bacterial and fungal pathogens (Asai *et al.*, 2002). On the other hand, several MAPKKs can activate the same MAPKs: for example, in yeast two-hybrid assays, MKK1 and MKK2 both interacted with a MAPK, MPK4 (Lee *et al*., 2008). It is possible that plants need such a large number of proteins in MAPK signaling to fine tune their responses to environmental stresses.

As a model plant, the solanaceous species, *Nicotiana attenuata*, has been intensively for its ecological interaction with herbivores for more than fifteen years. The germination of *N. attenuata* is induced by certain components of smoke after fire, and thus, this plant is one of the pioneers growing in its natural environment, the Great Basin Desert in Utah, USA. After germination, *N. attenuata* is often attacked by the larvae of the moth species *Manduca sexta* and other lepidopteran caterpillars. Therefore *N. attenuata* has evolved to have a complex system of defense responses to counteract herbivory (Baldwin, 1998).

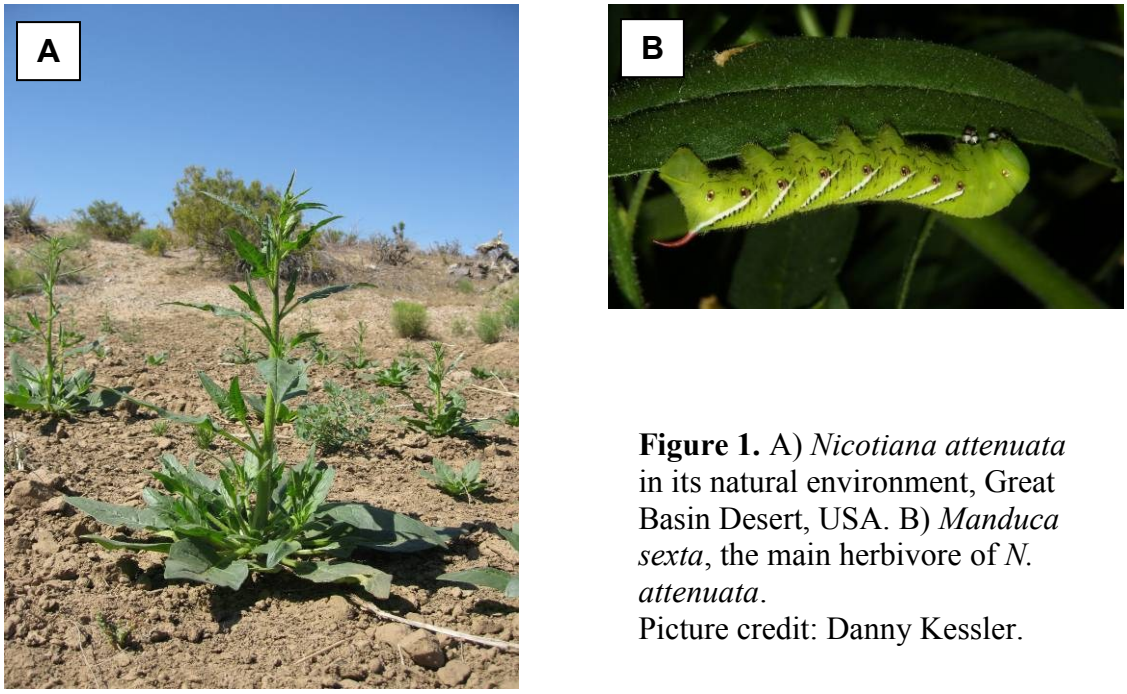

Figure 1. A) *Nicotiana attenuata* in its natural environment, Great Basin Desert, USA. B) *Manduca sexta*, the main herbivore of *N. attenuata*.
Picture credit: Danny Kessler.

Defense responses against herbivores are classified into indirect and direct defenses. Indirect defenses are traits that attract predators or parasitoids of the herbivores and thereby reduce the herbivore loads (Kessler & Baldwin, 2002). After herbivore attack, volatile organic compounds (VOCs) released from plants attract predators and parasitoids in laboratory setups (Dicke & van Loon, 2000) and in field studies (Kessler & Baldwin, 2001). Direct defenses are any traits (e.g. thorns, silica, trichomes, and secondary metabolites) that affect the susceptibility and/or the performance of attacking herbivores and thus increase plant fitness (Kessler & Baldwin, 2002). Secondary metabolites, e.g., alkaloids and phenolics, are generally believed to have functions in stress resistance, including herbivory (Duffey & Stout, 1996). They can be classified into three categories: 1) toxic compounds, 2) digestibility reducers and 3) antinutritive compounds (Duffey & Stout, 1996). Toxic compounds are for example alkaloids and phenolics. One of the best studied anti-herbivore toxic compounds in tobacco species is the pyridine alkaloid nicotine (Steppuhn *et al.*, 2004). Nicotine binds the acetylcholine receptors in the nervous systems, therefore it is extremely toxic to most animals, although some insects have evolved to be able to detoxify nicotine and even use it for their own defense (Kessler & Baldwin, 2002). Proteinase inhibitors have been shown to reduce the ability of herbivores to digest plant material, since they reduce the activity of midgut digestive proteinase enzymes, therefore decreasing larval growth rate and survivorship (Zavala *et al.*, 2008). Polyphenol oxidases are antinutritive enzymes that decrease the value of the wounded

plant by cross-linking proteins or catalyzing the oxidation of phenolic secondary metabolites to reactive and polymerizing quinines (Mayer, 2006; Mahanil *et al.*, 2008).

The regulation of defense responses is largely controlled by phytohormones. Phytohormones are small molecules which can appear in low concentrations but play a critical role in regulating plant development and responses to environmental stimuli, such as biotic or abiotic stresses (McCourt, 1999; Weyers & Paterson, 2001; Shakirova *et al.*, 2002; Nemhauser *et al.*, 2006; Lau & Deng, 2010). Known phytohormones are auxin, cytokinins, ethylene (ET), abscisic acid (ABA), gibberellic acids (GA), jasmonates (JAs, jasmonic acid and its derivatives), salicylic acid (SA), strigolactones, and brassinosteroids.

The main phytohormone involved in herbivore defense is JA (Wasternack, 2007; Howe & Browse, 2008; Jander, 2008; Wu & Baldwin, 2010; Howe, 2011). After its release from chloroplast membranes, linolenic acid is modified by lipoxygenase (Vick & Zimmerman, 1984), allene oxidase synthase (AOS), and allene oxide synthase (AOC), to form 12-oxo-phytodienoic acid (OPDA) (Vick & Zimmermann, 1979). JA is produced in peroxisomes, where OPDA is reduced by OPDA reductase 3 (OPR3) and thereafter three further oxidation reactions (Vick & Zimmerman, 1984; Stintzi *et al.*, 2001; Schaller *et al.*, 2004). *N. attenuata* plants attacked by *M. sexta* larvae generate a JA burst, which is induced by specific elicitor compounds, fatty acid-amino acid conjugates (FACs), in *M. sexta* oral secretions (OS), to activate herbivory-specific responses (Halitschke *et al.*, 2001). JA signaling is required to upregulate the activity of trypsin proteinase inhibitor (TPI) in the leaves (Zavala *et al.*, 2004) and to emit volatiles that attract predators of *M. sexta* (Kessler & Baldwin, 2001; Paschold *et al.*, 2007). As JA is the main phytohormone regulating defense responses of *N. attenuata*, it is important to understand how plant perceive herbivory and the regulatory mechanism underlying JA biosynthesis. Yet, no receptor has been identified, although the research on metabolism of FACs has provided a deeper insight on herbivore recognition (Bonaventure & Baldwin, 2010). It has been shown that two MAPKs, WIPK and SIPK, play an important role in the signaling cascade mediating FAC-induced JA biosynthesis (Wu *et al.*, 2007; Wu & Baldwin, 2010). In plant-herbivore interactions, none of the MAPK activating kinases, MAPK kinases (MAPKKs), was identified. **In Manuscript I, I identified two MAPKKs, NaMEK2 and NaMKK1, which regulate SIPK and WIPK in a different manner; NaMEK2 and NaMKK1 also control some aspects of the defense responses of *N. attenuata* against *M. sexta*. In Manuscript II, I identified three more MAPKKs, NaNPK2, NaMEK1 and NaSIPK, which do not activate SIPK, but influence TPI accumulation.**

Another group of kinases acting in the signaling cascades of plants are calcium-dependent protein kinases (CDPKs) (Yoon *et al.*, 1999; Romeis *et al.*, 2000; Romeis, 2001; Romeis *et al.*, 2001; Ivashuta *et al.*, 2005). The calcium ion is recognized as a secondary messenger in numerous plant signaling pathways. The information in [Ca^{2+}] changes is decoded by an array of Ca^{2+}-binding proteins resulting in changed gene expression and protein phosphorylation (Sanders *et al.*, 2002). Insect salivary secretions can increase the level of cytosolic Ca^{2+} levels, whereas mechanical damages do not (Maffei *et al.*, 2004). CDPKs constitute a large family of serine/threonine protein kinases. In *Arabidopsis thaliana* 34 CDPK genes have been found in its genome (Cheng *et al.*, 2002). CDPKs are a class of Ca^{2+} sensors, having both a protein kinase domain and a calmodulin-like domain (including an EF-hand Ca^{2+} binding site) in a single polypeptide (Klimecka & Muszynska, 2007). Upon insect attack, Arabidopsis CDPKs, CPK3 and CPK13, play a role in transcriptional activation of a plant defensive gene *PDF1.2* (Kanchiswamy *et al.*, 2010). In tobacco plants, NtCDPK2 was suggested to participate in the synthesis of ethylene and jasmonates and in cross-talk with the MAPK cascade activated by pathogen infection (Ludwig *et al.*, 2005).

In *N. attenuata*, four CDPK genes (*CDPK2*, *CDPK4*, *CDPK5* and *CDPK8*) have been shown to be upregulated after simulated herbivore attack (adding oral secretion of *M. sexta* (W+OS) to fresh wounds). Compared with empty vector control plants, in *SIPK*-silenced plants, the transcript levels of *CDPK2*, *CDPK4*, *CDPK5*, and *CDPK8* were altered (Wu *et al.*, 2007). This suggests a function of MAPKs in CDPK transcript regulation. Stably silencing *CDPK4* and *CDPK5* (*CDPK4/5*) leads to highly upregulated defense responses including elevated JA accumulation and high amount of secondary metabolites (Yang *et al*., in review). Moreover, plants silenced in *CDPK4/5* showed stunted growth, dark green leaves and had decreased fertility due to abortion of flower buds and flowers. These findings suggest that CDPK4/5 are not only repressors of *M. sexta*-induced defense responses, but might play an important role in plant development.

Classical phytohormones involved in plant development are auxin (IAA), gibberellic acids (GAs), cytokines, abscisic acid (ABA) and ethylene. Auxin influences the growth of plant organs by a stream that flows from the shoot apex to the tip of the root (Lomax *et al*., 1995). It has been shown that auxin promotes root growth of Arabidopsis by modulating cellular responses to GA (Fu & Harberd, 2003). Cytokines are mainly produced in the roots (Aloni *et al.*, 2004; Aloni *et al.*, 2005). From the sites of cytokine production, it moves in specific structural pathways and by different mechanisms to regulate plant development and differentiation (Aloni *et al.*, 2004; Aloni *et al.*, 2005). Abscisic acid regulates many processes

of plant growth and development, such as seed maturation and germination, seedling growth, flowering and stomatal movement, and is described as a key hormone mediating plant adaption to abiotic stresses like drought, salt and cold stress (Koornneef *et al.*, 1998; Leung & Giraudat, 1998; Finkelstein *et al.*, 2002; Cutler *et al.*, 2010). Ethylene is known to modulate Arabidopsis vegetative growth (Wang *et al.*, 2002; Achard *et al.*, 2006). Ethylene regulates vegetative growth, flowering, senescence process, and is also involved in biotic and abiotic stress resistance (Bleecker *et al.*, 2000).

Bioactive gibberellins control diverse aspects of plant growth and development, including seed germination, stem elongation, leaf expansion and flower and seed development (Yamaguchi, 2008). The GA metabolism has been studied intensively and most of the enzymes have been identified. GAs are biosynthesized from geranylgeranyl diphosphate (GGDP), a common C20 precursor for diterpenoids. In Arabidopsis, GA 20-oxidase (GA20ox) and GA 3β-hydroxylase (GA3ox) are encoded by a multi gene family. They catalyze the final steps in the formation of bioactive Gas (GA_1 and GA_4) and are downregulated by applying GA (Hedden & Phillips, 2000; Olszewski *et al.*, 2002). Plants having loss-of-function mutations in GA20ox and GA3ox are dwarf (Prat *et al.*, 2000; Sasaki *et al.*, 2002; Sun & Gubler, 2004). GAs are perceived by receptor, GID1 (GIBBERELLIN INSENSITIVE DWARF1), and the binding of bioactive GAs to GID1 induces degradation of DELLA proteins, which function as transcriptional repressors (Sun & Gubler, 2004). In addition to their function in plant growth regulation, Navarro *et al*. (2008) demonstrated that DELLA proteins modulate the balance between SA and JA signaling. Salicylic acid is another phytohormone, which is involved in pathogen resistance (Kunkel & Brooks, 2002). A growing body of evidence suggests that each phytohormone somehow influences the others to obtain the optimum output of plants fitness. It has not been elucidated yet, whether and how JA signaling influences the accumulation of GAs or GA signaling. **In Manuscript III, I analyzed the role of JA on plant development using CDPK4/5-silenced *N. attenuata*, and I demonstrate that GA biosynthetic gene expression is regulated by JA.**

Objectives of the thesis:

1. **Identify MAPKKs in *N. attenuata* and analyze their role in defense responses against *M. sexta***
2. **Analyze the function of JA and CDPK4/5 in the development of *N. attenuata***

Chapter 2: Manuscript overview

Manuscript 1

Two mitogen-activated protein kinase kinases, MKK1 and MEK2, are involved in wounding- and specialist lepidopteran herbivore *Manduca sexta*-induced responses in *Nicotiana attenuata*

Maria Heinrich, Ian T. Baldwin and Jianqiang Wu

Published in *Journal of Experimental Botany*, 62:12 (2011)

In Manuscript 1, I identified two mitogen-activated protein kinase kinases (MAPKKs), which are involved in the herbivore response against *Manduca sexta* in *Nicotiana attenuata*. We used a virus-induced gene silencing (VIGS) system to transiently silence these two MAPKKs, NaMEK2 and NaMKK1. In these genetically modified plants, I studied the function of MAPKKs in modulating phytohormone accumulation, the biosynthesis of secondary metabolites, and the activity of trypsin proteinase inhibitor (TPI). We found that NaMEK2, but not NaMKK1, has a high impact on the regulation of the MAPK, SIPK.

Maria Heinrich and Dr. Jianqiang Wu planned and performed the experiments, analyzed the data and wrote the manuscript. Prof. Ian T. Baldwin participated in the design and coordination of the study and wrote the manuscript.

Manuscript 2

Three MAPK Kinases, MEK1, SIPKK, and NPK2, are not Involved in Activation of SIPK after Wounding and Herbivore Feeding but Important for Accumulation of Trypsin Proteinase Inhibitors

Maria Heinrich, Ian T. Baldwin and Jianqiang Wu

Published in *Plant Molecular Biology Reporter*, doi:10.1007/s11105-011-0388-0 (2011)

In Manuscript 2, I identified three MAPKKs, NaMEK1, NaNPK2 and NaSIPKK, which are not involved in the regulation of the MAPK, SIPK, but are important for the accumulation of trypsin proteinase inhibitor (TPI). I used a virus-induced gene silencing (VIGS) system to transiently silence these MAPKKs. It was found that NaSIPKK and NaMEK1 influence the accumulation of the precursor of jasmonic acid (JA), 12-oxo-phytodienoic acid (OPDA), whereas silencing NaSIPKK enhanced the levels of wounding- and herbivory- induced JA. We suggest that NaSIPKK is involved in JA biosynthesis after herbivore attack without activating NaSIPK.

Maria Heinrich and Dr. Jianqiang Wu planned and performed the experiments, analyzed the data and wrote the manuscript. Prof. Ian T. Baldwin participated in the design and coordination of the study and wrote the manuscript.

Manuscript 3

Jasmonic acid specifically suppresses the transcript accumulation of *GA20ox* gene and thus inhibits the biosynthesis of gibberellic acid in the stem of irCDPK4/5 plants

Maria Heinrich, Christian Hettenhausen, Theodor Lange, Hendrik Wünsche, Jing-Jing Fang, Ian T. Baldwin and Jianqiang Wu

Manuscript in preparation, will be submitted to PLoS Biology

In Manuscript 3, I used irCDPK4/5 plants, which were stably silenced in calcium-dependent protein kinase 4 and 5 (CDPK4/5) and had highly increased levels of jasmonic acid (JA), to study the function of JA on the development of *N. attenuata* stem growth. Genetic analysis indicated that both the stunted stem elongation and the highly accumulated secondary metabolites resulted from the high contents of JA in irCDPK4/5 stems. Using quantitative real time-PCR (qRT-PCR), I found that the transcript levels of most GA biosynthetic genes were down-regulated in irCDPK4/5 stems, especially those of *GA20ox*, which is one of the most critical genes in gibberellin biosynthesis. Furthermore, abolishing JA biosynthesis or signaling in irCDPK4/5 plants restored normal transcript levels of GA biosynthetic genes, indicating that JA signaling represses the biosynthesis of gibberellins by inhibiting the transcript accumulation of GA biosynthetic genes. Consistently, applying a bioactive gibberellin, GA_3, to irCDPK4/5 plants largely restored stem elongation, confirming that gibberellin deficiency accounted for the stunted elongation of irCDPK4/5 stems. I further verified the function of *GA20ox* in stem elongation using a virus-induced gene silencing approach. This work demonstrates a suppressive function of JA signaling on the biosynthesis of gibberellins and shed light on the complex cross-talks between phytohormones.

Maria Heinrich and Dr. Jianqiang Wu planned and performed the experiments, analyzed the data and wrote the manuscript. Christian Hettenhausen helped with phytohormone analysis, virus-induced gene silencing and discussion of the results. Prof. Dr. Theodor Lange from the University of Braunschweig analyzed GA contents and quantified GAs and their precursors. Hendrik Wünsche helped with gibberellic acid treatment and virus-induced gene silencing. Jing-Jing Fang performed the NMR analysis of unknown compounds and identified the new

compound. Prof. Ian T. Baldwin participated in the design and coordination of the study and wrote the manuscript.

Chapter 3:

Two mitogen-activated protein kinase kinases, MKK1 and MEK2, are involved in wounding- and specialist lepidopteran herbivore *Manduca sexta*-induced responses in *Nicotiana attenuata*

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RESEARCH PAPER

Two mitogen-activated protein kinase kinases, MKK1 and MEK2, are involved in wounding- and specialist lepidopteran herbivore *Manduca sexta*-induced responses in *Nicotiana attenuata*

Maria Heinrich, Ian T. Baldwin and Jianqiang Wu*

Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Hans-Knöll Str. 8, D-07745 Jena, Germany

* To whom correspondence should be addressed. E-mail: jwu@ice.mpg.de

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Abstract

In a wild tobacco plant, *Nicotiana attenuata*, two mitogen-activated protein kinases (MAPKs), salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), play central roles in modulating herbivory-induced phytohormone and anti-herbivore secondary metabolites. However, the identities of their upstream MAPK kinases (MAPKKs) were elusive. Ectopic overexpression studies in *N. benthamiana* and *N. tabacum* suggested that two MAPKKs, MKK1 and MEK2, may activate SIPK and WIPK. The homologues of *MKK1* and *MEK2* were cloned in *N. attenuata* (*NaMKK1* and *NaMEK2*) and a virus-induced gene silencing approach was used to knock-down the transcript levels of these *MAPKK* genes. Plants silenced in *NaMKK1* and *NaMEK2* were treated with wounding or simulated herbivory by applying the oral secretions of the specialist herbivore *Manduca sexta* to wounds. MAPK activity assay indicated that after wounding or simulated herbivory NaMKK1 is not required for the phosphorylation of NaSIPK and NaWIPK; in contrast, NaMEK2 and other unknown MAPKKs are important for simulated herbivory-elicited activation of NaSIPK and NaWIPK, and after wounding NaMEK2 probably does not activate NaWIPK but plays a minor role in activating NaSIPK. Consistently, NaMEK2 and certain other MAPKKs, but not NaMKK1, are needed for wounding- and simulated herbivory-elicited accumulation of jasmonic acid (JA), JA–isoleucine, and ethylene. Furthermore, both NaMEK2 and NaMKK1 regulate the levels of trypsin proteinase inhibitors. The findings underscore the complexity of MAPK signalling pathways and highlight the importance of MAPKKs in regulating wounding- and herbivory-induced responses.

Key words: Defence, ethylene, herbivory, jasmonic acid, mitogen-activated protein kinase kinase, trypsin proteinase inhibitors.

Introduction

Mitogen-activated protein kinase (MAPK) cascades play critical roles in regulating various cellular processes in eukaryotes (Herskowitz, 1995; Chang and Karin, 2001; MAPK Group, 2002). They are located downstream of receptors and sensors and control cell physiology in response to various intra- and extracellular stimuli. The highly conserved MAPK cascades are composed of three kinases: MAPKs are phosphorylated by MAPK kinases (MAPKKs) at the threonine and tyrosine residues located in the activation loop (T-loop) between subdomains VII and VIII of the kinase catalytic domain; these MAPKKs are activated by the triple kinases, MAP kinase kinase kinases (MAPKKKs). Activated (phosphorylated) MAPKs can directly phosphorylate certain downstream targets, which include mainly transcription factors which, in turn, initiate stimulus-induced transcriptional changes,

Abbreviations: CP, caffeoylputrescine; DTG, 17-hydroxygeranyllinalool diterpene glycoside; HPLC-MS/MS, high-performance liquid chromatography–tandem mass spectrometry; MAPK, mitogen-activated protein kinase; SIPK, salicylic acid-induced protein kinase; TPI, trypsin proteinase inhibitor; VIGS, virus-induced gene silencing; WIPK, wound-induced protein kinase.

enzymes, and even proteins that function in cytokinesis (Chang and Karin, 2001; Liu *et al.*, 2004; Sasabe and Machida, 2006; Beck *et al.*, 2010).

Although the functions of MAPK cascades have been intensively studied in animals and yeast, how MAPK signalling is involved in plant development and stress responses is still not well understood. In plants, MAPK signalling pathways are important for development (Bergmann *et al.*, 2004; Lukowitz *et al.*, 2004; HC Wang *et al.*, 2007; Rodriguez *et al.*, 2010), responses to abiotic stresses, such as drought and salt (Kiegerl *et al.*, 2000; Kovtun *et al.*, 2000; Cardinale *et al.*, 2002; Xiong and Yang, 2003), and resistance to viral, bacterial, and fungal pathogens (reviewed in Pedley and Martin, 2005; Rodriguez *et al.*, 2010).

Emerging evidence has also indicated the involvement of MAPK signalling in plant defence against herbivores (Kandoth *et al.*, 2007; Wu *et al.*, 2007). In *Nicotiana attenuata*, attack from its natural herbivore, *Manduca sexta*, induces a myriad of responses on transcriptomic, proteomic, and metabolomic levels (Wu and Baldwin, 2010). *Nicotiana attenuata* recognizes the fatty acid–amino acid conjugates (FACs) in *M. sexta* oral secretions (OS) that are introduced into wounds during feeding and rapidly activates two MAPKs, salicylic acid-induced kinase (NaSIPK) and wound-induced protein kinase (NaWIPK); importantly, these kinases are required for the herbivory-induced biosynthesis of jasmonic acid (JA) and ethylene (Wu *et al.*, 2007). The central role of JA in plant defence against herbivores has been well documented (reviewed in Wasternack, 2007; Howe and Jander, 2008; Wu and Baldwin, 2010). JAR (JASMONATE RESISTANT) proteins conjugate JA with isoleucine to form JA–Ile (Staswick and Tiryaki, 2004), which binds to the COI1 receptor and thus activates most of the JA-induced responses (Chini *et al.*, 2007; Thines *et al.*, 2007), including biosynthesis of various defensive compounds, such as direct defensive compounds, trypsin proteinase inhibitors (TPIs) (Zavala *et al.*, 2004), caffeoylputrescine (CP) (Kaur *et al.*, 2010), nicotine (Steppuhn *et al.*, 2004), and diterpene glycosides (DTGs) (Jassbi *et al.*, 2008; Heiling *et al.*, 2010), and indirect defensive compounds, such as *trans*-α-bergamotene (Kessler and Baldwin, 2001). In *N. attenuata*, *M. sexta* attack, but not mechanical wounding, induces a burst of ethylene; genetic analysis indicated that ethylene is important for herbivory-induced nicotine production (von Dahl *et al.*, 2007). Yet little is known about the signalling pathway that transduces FAC recognition into MAPK activation and eventually JA production in plants.

The *Arabidopsis* genome harbours ~60 MAPKKKs, 10 MAPKKS, and 20 MAPKs (MAPK Group, 2002). The small number of MAPKKs suggests that MAPKKs may have multiple MAPK targets and that interactions among different signalling pathways are concentrated at the level of MAPKKs (MAPK Group, 2002; Hamel *et al.*, 2006; Andreasson and Ellis, 2010). A growing body of evidence has revealed the important functions of MAPKKs in plant development and stress-induced responses. In tobacco, NQK1/NtMEK1 is required for cell cytokinesis (Soyano *et al.*, 2003). The *Arabidopsis* double mutant *mkk4 mkk5* develops densely clustered stomata and is seedling lethal, demonstrating the important role of MAPKKs in development (HC Wang *et al.*, 2007). Detached leaves of an *mkk9* mutant have delayed senescence (Zhou *et al.*, 2009). Several MAPKKs are involved in abiotic stress responses (Kiegerl *et al.*, 2000; Teige *et al.*, 2004; Gomi *et al.*, 2005; Xu *et al.*, 2008), and resistance to pathogens (Asai *et al.*, 2002; Jin *et al.*, 2003; Liu *et al.*, 2004; Meszaros *et al.*, 2006; Doczi *et al.*, 2007; Takahashi *et al.*, 2007). In *Arabidopsis*, after perception of pathogen elicitor flg22, AtMKK4 and AtMKK5 activate AtMPK3 and AtMPK6, the homologues of *Nicotiana* WIPK and SIPK, respectively (Asai *et al.*, 2002). Furthermore, overexpressing the constitutively active form of NtMEK2 (the homologue of AtMKK4/AtMKK5) in tobacco leads to activation of NtSIPK and NtWIPK (Yang *et al.*, 2001; Jin *et al.*, 2003). Overexpression of another *Arabidopsis* MAPKK, AtMKK9, activates AtMPK3 and AtMPK6 in protoplasts, and this MAPK cascade mediates the stability of EIN3 (ETHYLENE INSENSITIVE3), an important component in ethylene signalling (Yoo *et al.*, 2008). Furthermore, *Arabidopsis* plants overexpressing AtMKK9 have enhanced ethylene and camalexin levels (Xu *et al.*, 2008). In *N. benthamiana*, a close homologue of *Arabidopsis* AtMKK9, NbMKK1, interacts with NbSIPK in yeast, and ectopically overexpressing NbMKK1 activates NbSIPK (Takahashi *et al.*, 2007).

In *N. attenuata*, NaSIPK and NaWIPK are pivotal MAPKs that regulate plant responses to herbivory. However, their upstream MAPKKs involved in herbivore defence responses were unknown. Using a reverse genetic approach, the transcript levels of two *MAPKK* genes, *NaMEK2* and *NaMKK1*, were knocked down and it was found that NaMEK2 is important in mediating *M. sexta* herbivory-induced defence responses, while NaMKK1 plays only a minor role. After simulated herbivory, NaMEK2 and certain other MAPKKs, but not NaMKK1, are required for the activation of NaSIPK and NaWIPK, and thus JA and ethylene biosynthesis. The data highlight the important roles of MAPKKs in plant–herbivore interaction and the complexity of the regulation of JA and ethylene biosynthesis.

Materials and methods

Molecular cloning and virus-induced gene silencing (VIGS)

Nicotiana attenuata NaMKK1 and *NaMEK2* (GenBank accession numbers: HQ023234 and HQ023235) were amplified using Phusion DNA polymerase (Finnzymes Oy, Espoo, Finland) (primer sequences are listed in Supplementary Table S1 available at *JXB* online) and the purified PCR products were cloned into pJET1.2 vector (Fermentas GmbH, St. Leon-Rot, Germany) and sequenced. Partial *NaMEK2* and *NaMKK1* sequence were amplified using plasmids as templates and gene-specific primers (listed in Supplementary Table S2). The PCR products were digested with appropriate restriction endonucleases and were further ligated into pTV00 to obtain the constructs pTV-NaMEK2 and pTV-NaMKK1.

Agrobacterium tumefaciens carrying these constructs was inoculated into plants to obtain VIGS (virus-induced gene silencing)

plants following a procedure optimized for *N. attenuata* (Saedler and Baldwin, 2004). Plants inoculated with *A. tumefaciens* carrying pTV00 (empty vector) were used for comparisons (EV plants). Plants silenced in *NaPDS* (*phytoene desaturase*) were used to monitor the degree of VIGS visually, since these plants showed a photo-bleaching phenotype (Saedler and Baldwin, 2004). About 14 d after inoculation, when the leaves of *NaPDS*-silenced plants were completely white, experiments were performed.

Phylogenetic analysis of MAPKKs

MAPKK protein sequences were deduced from their respective nucleotide sequences (accession numbers are listed in Supplementary Table S3 at *JXB* online). Protein sequences were aligned using the Clustal W algorithm (DNAStar Inc., Madison, WI, USA). An unrooted Neighbor–Joining tree and bootstrap analysis (1000 replications) were conducted using MEGA 4 software (Tamura *et al.*, 2007).

Plant growth and treatments

Plants of the 31st generation of an *N. attenuata* inbred line were used in all experiments. Plants were grown at 22 °C under 16 h of light in a growth chamber. In all the experiments, leaves of rosette-stage (~4–5 weeks old) plants were used. Wounding was performed by rolling a fabric pattern wheel three times on each side of the midvein. The wounded leaves were immediately supplied with either 15 µl of water (W+W) or 15 µl of 1:5 diluted OS from *M. sexta.* For the collection of *M. sexta* OS, larvae were reared on *N. attenuata* wild-type plants until the third to fifth instar. OS were collected on ice as described in Roda *et al.* (2004).

Manduca sexta *growth bioassays*

Manduca sexta eggs from in-house reared populations were kept in a growth chamber (Snijders Scientific, Tilburg, The Netherlands) at 26 °C under 16 h of light, and at 24 °C in 8 h of darkness, until larvae hatched. Freshly hatched *M. sexta* neonates were placed on fully developed leaves of 30 replicated rosette-stage NaMEK2-VIGS, NaMKK1-VIGS, and EV plants (one larva per plant). The larval masses were measured on day 5, 8, and 12.

Transcriptional analysis

Total RNA was extracted from leaves using the TRIzol reagent (Invitrogen, Paisley, UK). A 0.5 µg aliquot of total RNA of each sample was reverse-transcribed using oligo(dT)$_{12–18}$ and Superscript II reverse transcriptase (Invitrogen) following the manufacturer's instructions. Quantitative real-time PCR (qPCR) was carried out on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), using qPCR Core kits (Eurogentec, Liege, Belgium). *Elongation factor 1A* (*NaEF1A*) transcript levels were used to normalize total cDNA concentration variations. The sequences of primers used for qPCR are provided in Supplementary Table S4 at *JXB* online.

Phytohormone analysis

About 100 mg of frozen plant tissue were homogenized in 2 ml microcentrifuge tubes containing two metal balls and 1 ml of ethyl acetate spiked with 200 ng of D_2-JA, and 40 ng of D_4-salicylic acid (SA) and $^{13}C_6$-JA–Ile. Homogenization was done twice with 200 strokes min^{-1} for 1 min using a Geno/Grinder 2000 (SPEX CertiPrep, Metuchen, NJ, USA). Samples were centrifuged at 13 000 *g* for 20 min at 4 °C. The supernatants were dried on a vacuum concentrator (Eppendorf AG, Hamburg, Germany). The residues were resuspended in 500 µl of 70% methanol by vortexing for 5 min, and centrifuged for 10 min at 4 °C (13 000 *g*). Supernatants were transferred to crimp vials, and sample measurements were carried out as described in Wu *et al.* (2007). Ethylene emissions were measured on a photoacoustic spectrometer (INVIVO GmbH, Sankt Augustin, Germany) as described in von Dahl *et al.* (2007). Leaves of *N. attenuata* plants were treated with W+OS or left untreated for control. Immediately after treatments three leaves were weighed and enclosed in a three-neck 250 ml round-bottom glass flask for 5 h, and then the concentration of collected ethylene was measured.

Analysis of trypsin proteinase inhibitor activity

Trypsin proteinase inhibitor (NaTPI) activity was quantified using a radial diffusion assay protocol described by van Dam *et al.* (2001).

Protein extraction and in-gel kinase activity assay

The tissue of five replicates was pooled and ground in liquid nitrogen. About 100 mg of tissue were resuspended in 300 µl of extraction buffer [100 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM phenylmethylsulphonyl floride, 10% glycerol, and one proteinase inhibitor cocktail tablet per 10 ml of extraction buffer (Roche, Mannheim, Germany)]. Samples were then centrifuged at 4 °C, 13 000 *g* for 20 min and the supernatants were transferred to fresh tubes. Protein concentrations were measured using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (Sigma-Aldrich, Hamburg, Germany) as a standard. A 10 µg aliquot of total protein from each sample was used for in-gel kinase activity assay according to a procedure described by Zhang and Klessig (1997). The images of in-gel kinase activity assays were obtained on a phosphorimager (FLA-3000 phosphor imager system, Fuji Photo Film, Stamford, CT, USA), and the band intensities were quantified using the AIDA software (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

Statistical analysis

Data were analysed by unpaired *t*-tests using SPSS Statistics Version 17.0 (www.spss.com).

Results

Phylogenetic analysis of N. attenuata *NaMEK2 and NaMKK1*

Using sequences of *NtMEK2* in *N. tabacum* (Yang *et al.*, 2001; Zhang and Liu, 2001) and *NbMKK1* in *N. benthamiana* (Takahashi *et al.*, 2007) as references, the open reading frames of *NaMEK2* and *NaMKK1* in *N. attenuata* were cloned. Sequence alignments indicated that the protein sequences of NaMEK2 and NaMKK1 shared 99% and 95% similarity to that of NtMEK2 and NbMKK1, respectively, and the conserved motif sequence [S/TxxxxxS/T] of MAPKKs (MAPK Group, 2002) was also found in both kinases (Supplementary Fig. S1 at *JXB* online). Moreover, phylogenetic analysis of NaMEK2, NaMKK1, and MAPKKs in *A. thaliana*, *N. tabacum*, *N. benthamiana*, and *Oryza sativa* indicated that NaMEK2 is a close homologue of AtMKK4 and AtMKK5 (group C of MAPKKs) and NaMKK1 is closely related to AtMKK7, AtMKK8, and AtMKK9 (group D of MAPKKs) (MAPK Group, 2002) (Fig. 1). It is likely that both *NaMKK1* and *NaMEK2* are single genes in *N. attenuata*, since searching the tobacco expressed sequence tag (EST) database and an *N. attenuata* transcriptome database obtained by 454 sequencing revealed no other close

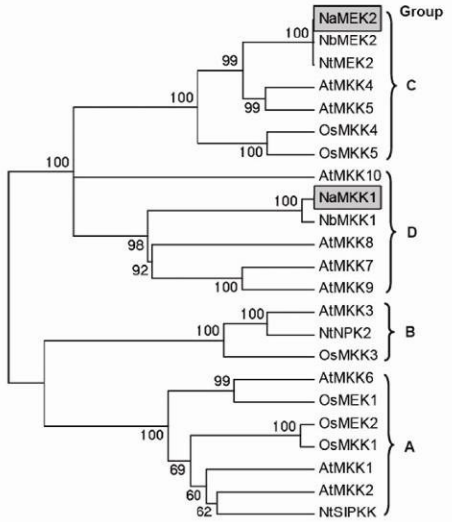

Fig. 1. Phylogenetic analysis of plant mitogen-activated protein kinase kinases (MAPKKs). Protein sequences of MAPKKs in *Arabidopsis*, *Nicotiana* spp., and rice were aligned using the Clustal W algorithm. An unrooted Neighbor–Joining tree and bootstrap analysis were performed with the MEGA 4 program. The species of origin of the MAPKKs are indicated by the abbreviation in front of the protein names: At, *Arabidopsis thaliana*; Na, *Nicotiana attenuata*; Nb, *Nicotiana benthamiana*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*. NaMKK1 and NaMEK2 are highlighted with grey backgrounds. Letters A to D represent different MAPKK groups (see MAPK Group, 2002).

homologues. However, the possibility that *NaMKK1* and *NaMEK2* have paralogues, which might have low expression levels or are expressed in specific organs or tissues, cannot be completely ruled out.

Transcriptional regulation of NaMKK1 *and* NaMEK2 *in* N. attenuata

To determine whether these two *MAPKK* genes are involved in herbivory-induced transcriptional responses, their transcriptional changes were examined in *N. attenuata* after wounding and simulated herbivory treatment. A fabric pattern wheel was rolled over *N. attenuata* leaves to generate puncture wounds; thereafter, 15 µl of water were immediately applied to wounds (W+W). To mimic herbivory, 15 µl of *M. sexta* OS were applied (W+OS) (Halitschke *et al.*, 2003). qPCR analyses indicated that the transcript levels of *NaMKK1* were elevated ~7-fold 1 h after W+W; while after W+OS treatment *NaMKK1* reached its highest level of transcription (10-fold increase) by 5 h (Fig. 2A). W+W treatment marginally enhanced the levels of *NaMEK2* transcripts, while *NaMEK2* transcript levels were elevated >25 times 1 h after W+OS (Fig. 2B). These transcript data suggest possible involvement of *NaMKK1* and *NaMEK2* in wounding and herbivore defence responses. Whether these differential transcriptional

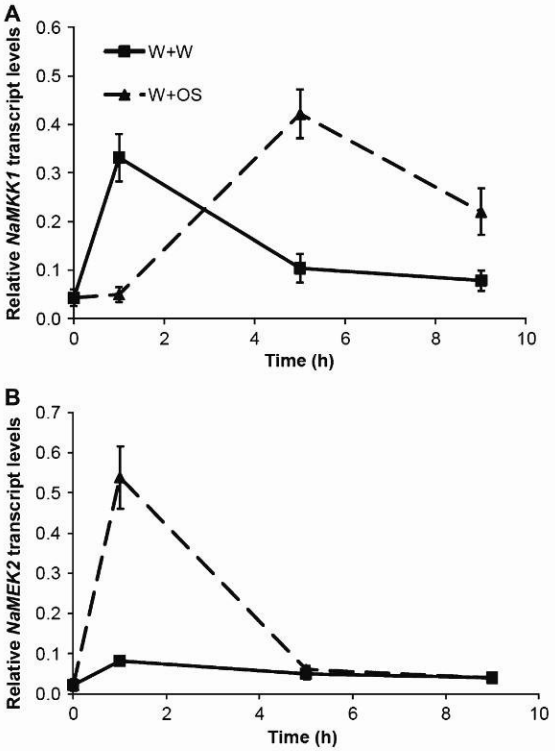

Fig. 2. Transcript levels of *NaMKK1* and *NaMEK2* after wounding and simulated herbivory. *Nicotiana attenuata* plants were wounded with a fabric pattern wheel, and 15 µl of water or *M. sexta* oral secretions (OS) were applied immediately to wounds (W+W and W+OS, respectively); untreated plants served as controls. Samples were harvested after1, 5, and 9 h and the transcript levels of (A) *NaMKK1* and (B) *NaMEK2* were analysed by qPCR.

regulations of *NaMKK1* and *NaMEK2* are further translated into different levels of NaMKK1 and NaMEK2 protein abundance/activity needs to be studied further.

NaMEK2, but not NaMKK1, is required for the phosphorylation of NaSIPK and NaWIPK after simulated herbivory

To study the function of NaMKK1 and NaMEK2 in wounding- and *M. sexta* herbivory-induced responses in *N. attenuata*, RNA interference (RNAi) constructs harbouring partial *NaMKK1* and *NaMEK2* sequences in an inverted repeat orientation were prepared and *N. attenuata* was transformed by *A. tumefaciens* carrying these constructs. Although stable RNAi lines of *NaMKK1*-silenced plants were obtained, after screening 20 independent lines transformed with the *NaMEK2*-RNAi construct, all 20 lines were found to be tetraploids. Therefore, a transient silencing approach, VIGS, was employed to knock-down the transcript levels of these two *MAPKK* genes. Plants inoculated with *A. tumefaciens* carrying pTV00, pTV-NaMKK1, and pTV-NaMEK2 formed EV,

NaMKK1-VIGS, and NaMEK2-VIGS plants, respectively. qPCR analyses indicated that compared with those in EV plants, *NaMKK1* and *NaMEK2* transcript levels were reduced 89% and 95% in NaMKK1-VIGS and NaMEK2-VIGS plants (Fig. 3A). Silencing either gene using VIGS did not result in obvious developmental abnormalities in *N. attenuata* (data not shown).

An in-gel kinase activity assay was performed to determine whether NaMKK1 and NaMEK2 are the upstream MAPKKs for NaSIPK and NaWIPK when plants are challenged with wounding and herbivory. Plants were treated with W+W and W+OS and samples were collected after 10 min and 30 min. When untreated, no obvious different levels of MAPK activity were detected among EV, NaMKK1-VIGS, and NaMEK2-VIGS plants (Fig. 3B, top panel). In all plants, W+W and W+OS treatment rapidly enhanced the activity of NaSIPK and NaWIPK (as early as 10 min), indicating that *N. attenuata* recognized *M. sexta* OS and responded with higher MAPK activity levels than those induced by just wounding (Wu *et al.*, 2007). Importantly, compared with EV, 10 min after W+W and 10 min and 30 min after W+OS, NaMEK2-VIGS plants showed ~50% reduced NaSIPK activity levels (Fig. 3B, middle panel; for quantification of band intensities see Supplementary Fig. S2A at *JXB* online). In-gel kinase assays revealed only weak NaWIPK activity even after inductions. NaMEK2-VIGS plants seemed to have decreased levels of NaWIPK activity after W+OS, but not after W+W (Fig. 3B, bottom panel); in contrast, silencing *NaMKK1* had no detectable effect on the activity levels of NaSIPK (and probably also NaWIPK) after either treatment.

Therefore, after mechanical wounding and simulated herbivory, NaMEK2 is important for the activation of NaSIPK, while NaWIPK seems to require NaMEK2 for phosphorylation only after simulated herbivory. Consistent results were obtained from an independently repeated experiment (Supplementary Fig. S2B at *JXB* online). These data suggest that very probably some other MAPKKs are also involved in the regulation of NaSIPK and NaWIPK in *N. attenuata*'s responses to wounding and herbivory, since silencing neither *NaMEK2* nor *NaMKK1* greatly compromises MAPK activity.

NaMEK2 but not NaMKK1 regulates wounding- and herbivory-induced accumulation of phytohormones in N. attenuata

Given that NaSIPK and NaWIPK are important regulators of wounding- and *M. sexta* herbivory-induced JA and JA–Ile accumulation and these phytohormones play a central role in mediating resistance to herbivores, whether silencing *NaMKK1* and *NaMEK2* alters the levels of JA and JA–Ile after these treatments was examined next. In EV plants, 1.5 h after W+W treatment JA reached 800 ng g^{-1} fresh mass (FM); consistent with the more highly augmented NaSIPK and NaWIPK activity levels after W+OS, EV plants accumulated up to 3600 ng g^{-1} FM of JA (Fig. 4A). The

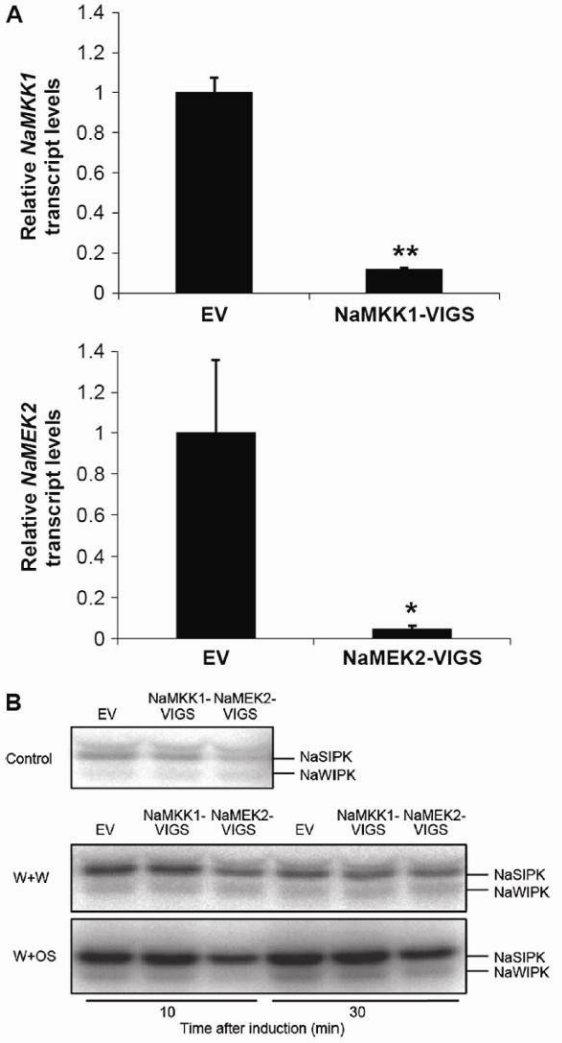

Fig. 3. MAPK activity in wounding- or simulated herbivory-induced responses in EV, NaMKK1-VIGS, and NaMEK2-VIGS plants. (A) *NaMKK1* and *NaMEK2* transcript levels are highly suppressed in NaMKK1-VIGS and NaMEK2-VIGS plants. Rosette leaves of EV, NaMKK1-VIGS, and NaMEK2-VIGS plants were harvested and the transcript levels of *NaMKK1* and *NaMEK2* were analysed by qPCR. The transcript levels of *NaMKK1* and *NaMEK2* in EV plants were designated as 1. Asterisks indicate significant differences between EV and NaMKK1-VIGS or NaMEK2-VIGS plants (*t*-test; $^{*}P$ <0.05; $^{**}P$ <0.01; n=5). (B) MAPK activity in EV, NaMKK1-VIGS, and NaMEK2-VIGS plants after wounding and simulated herbivory treatment. Plants were wounded with a fabric pattern wheel, and 15 μl of water or *M. sexta* oral secretions (OS) were applied immediately to wounds [W+W (middle panel) and W+OS (bottom panel), respectively]; untreated plants served as controls (top panel). Samples were harvested after 0, 10, and 30 min and immediately frozen in liquid nitrogen. MAPK activity was detected with an in-gel kinase assay.

JA–Ile contents in EV plants followed similar patterns: 1 h after treatments, W+OS induced 4-fold higher JA–Ile levels than did W+W treatment (Fig. 4B). Congruent with their MAPK activity levels, NaMKK1-VIGS plants had no different JA and JA–Ile levels compared with EV plants after W+W and W+OS treatment, whereas NaMEK2-VIGS exhibited ~50% lower levels of JA and JA–Ile at most of the time points examined after either treatment (Fig. 4).

Since wounding barely elicits ethylene biosynthesis, ethylene production was only measured in W+OS-elicited plants (von Dahl *et al.*, 2007). After W+OS treatment, the same amount of ethylene was detected in NaMKK1-VIGS and EV plants, and NaMEK2-VIGS plants had 40% reduced ethylene emissions (Fig. 4C).

Many studies have indicated that SA suppresses JA accumulation (Spoel *et al.*, 2003; Cipollini *et al.*, 2004; Leon-Reyes *et al.*, 2010). To rule out the possibility that the decreased JA levels in NaMEK2-VIGS plants resulted from augmented SA levels in these plants, SA contents were quantified. EV, NaMKK1-VIGS, and NaMEK2-VIGS plants showed no difference in basal and W+W-induced SA levels (Supplementary Fig. S3 at *JXB* online). After W+OS treatment, SA levels in *NaMKK1*- and *NaMEK2*-silenced plants were not higher than those of EV plants and tended to

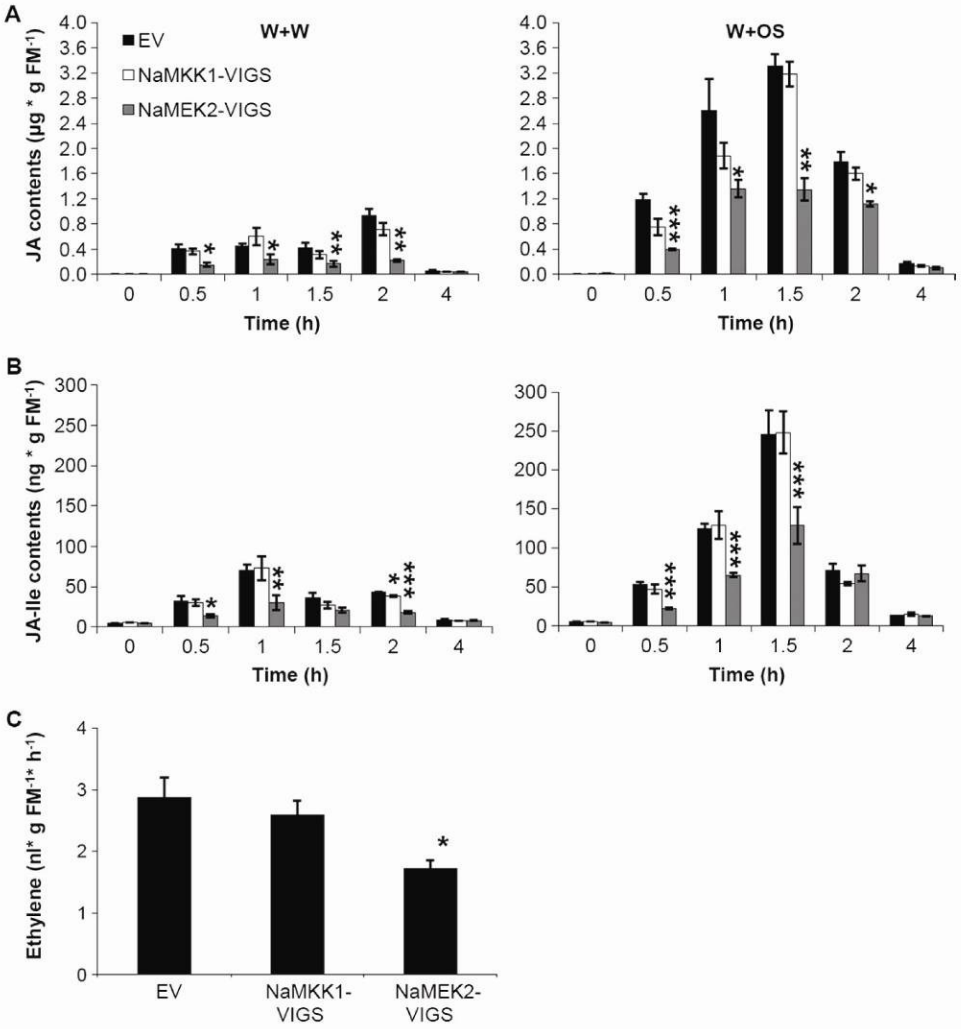

Fig. 4. Silencing *NaMKK1* and *NaMEK2* decreases wounding- or simulated herbivory-induced levels of phytohormones. (A) and (B) EV, NaMKK1-VIGS, and NaMEK2-VIGS plants were wounded with a fabric pattern wheel, and 15 µl of water or *M. sexta* oral secretions (OS) were applied immediately to wounds (W+W and W+OS, respectively). Samples were harvested after the indicated times. Contents (mean ±SE) of JA (A) and JA–Ile (B) were measured with HPLC-MS/MS. (C) EV, NaMKK1-VIGS and NaMEK2-VIGS plants were treated with W+OS, and ethylene accumulated in 5 h was collected and analysed. Asterisks indicate significant differences between EV and NaMKK1-VIGS or NaMEK2-VIGS plants (*t*-test; **P* <0.05; ***P* <0.01; ****P* <0.001; *n*=5).

be lower by 50% in *NaMEK2*-silenced plants 1 h after W+OS (Supplementary Fig. S3).

Thus, NaMEK2 but not NaMKK1 is important for the regulation of wounding- and *M. sexta* herbivory-induced defence-related phytohormones.

Silencing NaMKK1 and NaMEK2 in N. attenuata *compromises NaTPI activity*

TPIs are important anti-herbivore compounds in Solanaceae, including *N. attenuata* (Ryan, 1989; Haq *et al.*, 2004; Zavala *et al.*, 2004). To analyse the function of NaMKK1 and NaMEK2 in regulating NaTPI, the activity of NaTPI was determined in EV, NaMKK1-VIGS, and NaMEK2-VIGS plants 3 d after they were treated with W+W or W+OS (non-treated plants served as controls). No significant differences were found among the non-treated samples (Fig. 5A). However, after W+W treatment, compared with EV, NaMKK1-VIGS plants showed ~60% decreased NaTPI activity levels, while NaMEK2-VIGS plants had similar levels. Furthermore, W+OS-treated NaMKK1-VIGS and NaMEK2-VIGS exhibited ~40% and 50% lower levels of NaTPI activity, respectively (Fig. 5A).

In *N. attenuata*, nicotine, CP, and DTGs are also important defensive compounds against *M. sexta* larvae. Neither W+W nor W+OS treatment elevated the contents of these compounds even in EV plants (data not shown). Very probably this was caused by the low temperature required for efficient VIGS (Kaplan *et al.*, 2004; Shohael *et al.*, 2006).

Bioassays were performed to examine whether knocking down *NaMKK1* and *NaMEK2* alters the performance of the specialist herbivore *M. sexta*. Freshly hatched neonates were placed on EV, NaMKK1-VIGS, and NaMEK2-VIGS plants, and their masses were recorded over 12 d. Despite the decreased NaTPI activity in NaMKK1-VIGS and NaMEK2-VIGS, *M. sexta* larvae gained similar mass on all plants (Fig. 5B).

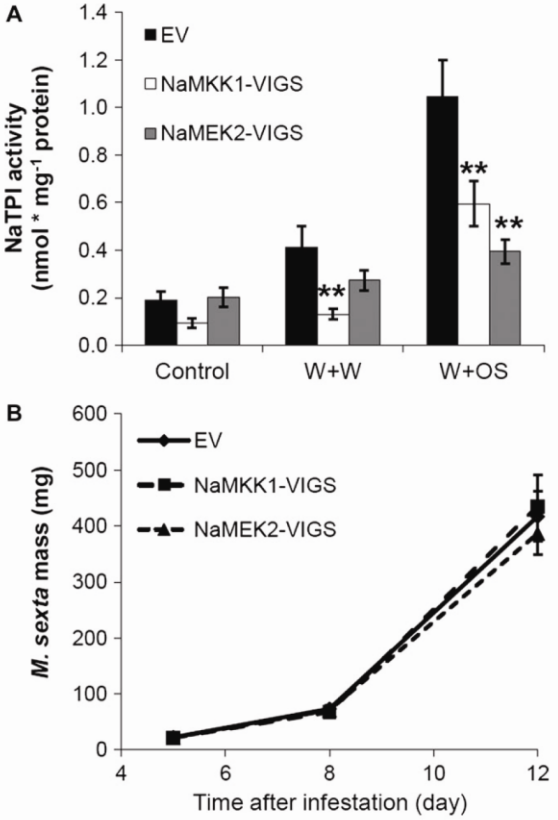

Fig. 5. NaMKK1-VIGS and NaMEK2-VIGS plants have decreased NaTPI activity, but do not exhibit compromised resistance to *M. sexta*. (A) NaTPI activity in EV, NaMKK1-VIGS, and NaMEK2-VIGS plants. Plants were wounded with a fabric pattern wheel, and 15 μl of water or *M. sexta* oral secretions (OS) were applied immediately to wounds (W+W and W+OS, respectively); untreated plants served as controls. Three days after treatments, samples were collected and NaTPI activity was analysed. Asterisks indicate significant differences between EV and NaMKK1-VIGS or NaMEK2-VIGS plants (*t*-test; **P* <0.05; ***P* <0.01; *n*=5). (B) *Manduca sexta* mass gain on EV, NaMKK1-VIGS, and NaMEK2-VIGS plants. Each type of plant was infested with 30 *M. sexta* neonates (one larva per plant) and larval masses (mean ±SE) were measured after 5, 8, and 12 d.

Discussion

MAPKs play central roles in the activation of plant defence responses against abiotic and biotic stresses (Bergmann *et al.*, 2004; Lukowitz *et al.*, 2004; Pedley and Martin, 2005; Andreasson and Ellis, 2010; Rodriguez *et al.*, 2010; HC Wang *et al.*, 2007). At least two MAPKs, SIPK and WIPK, are important for plant resistance to herbivores (Wu *et al.*, 2007). However, the identities of their upstream MAPKKs in plant–herbivore interactions were largely unknown. Using a reverse genetic approach, it is shown here that two MAPKKs, NaMKK1 and NaMEK2, are involved in wounding- and *M. sexta* feeding-induced responses in *N. attenuata*.

NaMEK2, but not NaMKK1, is upstream of NaSIPK and NaWIPK

Thus far, only two MAPKs, NaSIPK and NaWIPK and their homologues in tomato, are known to play a role in resistance to herbivore attack (Kandoth *et al.*, 2007; Wu *et al.*, 2007). Whether AtMPK6 and AtMPK3 (homologues of NaSIPK and NaWIPK in *Arabidopsis*) are also important for herbivory-induced responses is still unclear.

Using ectopic overexpression systems, a few studies have demonstrated that in *Arabidopsis* AtMKK4 and AtMKK5 phosphorylate AtMPK6 and AtMPK3 (Asai *et al.*, 2002), and their close homologue in tobacco, NtMEK2, activates NtSIPK and NtWIPK (Yang *et al.*, 2001; Zhang and Liu, 2001). Consistent with an AtMKK4/AtMKK5–AtMPK6/AtMPK3 and NtMEK2–NtSIPK/NtWIPK cascade, NaMEK2 was identified to be located upstream of NaSIPK and NaWIPK in the herbivory-induced signalling pathway using a knock-down approach. Importantly,

silencing *NaMEK2* only reduced NaSIPK activity levels after W+OS treatment by ~50%, although qPCR analysis indicated that only 5% of *NaMEK2* transcript levels were detected in NaMEK2-VIGS plants. It is speculated that one or more other unknown MAPKKs also phosphorylate NaSIPK when *N. attenuata* is challenged by *M. sexta* feeding. The possibility that NaMEK2-VIGS plants had substantially greater levels of NaMEK2 protein than the levels of *NaMEK2* transcripts cannot be ruled out. In the wounding-activated signalling pathway, NaWIPK seems not to be located downstream of NaMEK2, given that *NaMEK2*-silenced plants did not have noticeably altered NaWIPK activity after wounding. Given the low activity of NaWIPK in in-gel kinase assays, this should be confirmed with immunocomplex kinase activity assays using an NaWIPK-specific antibody. Furthermore, in contrast to its reduced activity after simulated herbivory (at least by 30 min), the activity of NaSIPK in *NaMEK2*-silenced plants decreased only shortly after wounding (10 min) but regained the levels found in EV plants by 30 min. These data suggest that wounding and herbivory may activate overlapping but distinct MAPK pathways involving different MAPKKs, given that NaMEK2 is important for herbivory-induced activation of NaSIPK (and probably NaWIPK), but in response to wounding it only plays a minor role. Identification of the other MAPKK (or MAPKKs) that activate NaSIPK and NaWIPK will provide valuable insight into the mechanism by which plants distinguish mechanical wounding and herbivory and thus deploy appropriate defences.

Overexpression of *NbMKK1* results in phosphorylation of NbSIPK in *N. benthamiana* (Takahashi *et al.*, 2007). Transiently expressing AtMKK9 (a close homologue of NaMKK1) in tobacco and *Arabidopsis* leads to SIPK/AtMPK6 and WIPK/AtMPK3 activation (Xu *et al.*, 2008), and transforming *Arabidopsis* protoplasts with a constitutively active form of AtMKK9 results in phosphorylation of AtMPK6 and AtMPK3 (Yoo *et al.*, 2008). However, silencing experiments indicated that NaMKK1 is not required for the phosphorylation of NaSIPK and NaWIPK after wounding and simulated herbivory. It is very unlikely that the silencing of *NaMKK1* was not sufficient to knock-down the protein levels of NaMKK1 since, after wounding and simulated herbivory treatment, *NaMKK1*-silenced plants exhibited compromised NaTPI activity. It is speculated that this was because ectopic overexpression may have led to very high levels of protein and thus produced non-physiological interactions. It is also possible that NaMKK1 activates NaSIPK and NaWIPK in other stimulus-activated signalling cascades, such as those elicited by pathogens (Takahashi *et al.*, 2007).

Function of NaMEK2 in wounding- and herbivory-induced biosynthesis of phytohormones

SIPK and WIPK are important regulators of wounding- and herbivory-induced JA (and JA–Ile) accumulation (Kandoth *et al.*, 2007; Wu *et al.*, 2007). Consistent with this, *NaMEK2*-silenced plants had nearly 50% decreased JA and JA–Ile levels after both wounding and simulated herbivory. NaMKK1 is not required for the phosphorylation of NaSIPK and NaWIPK after wounding and herbivory; congruently, no changes of JA and JA–Ile levels were detected in *NaMKK1*-silenced plants. Moreover, compared with those in EV, no large differences in SA levels were found in *NaMKK1*- and *NaMEK2*-silenced plants. Kobayashi *et al.* (2010) examined the function of SIPK and WIPK in modulating the levels of JA and SA after *N* gene-carrying tobacco plants were challenged with tobacco mosaic virus (TMV): simultaneously silencing *SIPK* and *WIPK* highly compromises TMV viral accumulation, and this is associated with increased SA and decreased JA contents. It is speculated that silencing *MKK1* in tobacco may not affect viral amplification and the accumulation of JA and SA, but *MEK2*-silenced plants could somewhat resemble plants silenced in both *SIPK* and *WIPK* (TMV amplification and the accumulation of JA and SA).

An elegant study revealed that *Arabidopsis* AtMPK6 phosphorylates 1-aminocyclopropane-1-carboxylic acid synthases (AtACS2 and AtACS6) and thus stabilizes these enzymes and greatly enhances ethylene biosynthesis (Liu and Zhang, 2004). However, after application of a pathogen elicitor, an *mpk6* null mutant still produces 50% of the amount of ethylene synthesized by wild-type plants (Liu and Zhang, 2004). Similarly, silencing *NaSIPK* in *N. attenuata* results in 40% reduced ethylene production after simulated herbivory treatment (Wu *et al.*, 2007). These facts suggest that certain AtMPK6/NaSIPK-independent pathways regulate the other 50% of ethylene production after pathogen/herbivory elicitation. Notably, even though the activity of NaSIPK in NaMEK2-VIGS plants is not decreased to the same extent as that in NaSIPK-VIGS plants or in *mpk6* null mutants (Liu and Zhang, 2004; Wu *et al.*, 2007), ethylene production is still 40% reduced. Either full activation of NaSIPK is critical for herbivory-induced ethylene biosynthesis or, in addition to NaSIPK, NaMEK2 also phosphorylates another MAPK, which also regulates ethylene production. Identification of this NaSIPK-independent ethylene regulation pathway will provide valuable insight into the mechanism by which plants control the biosynthesis of this important hormone.

NaMKK1 and NaMEK2 regulate the defence metabolite NaTPI

TPIs play an important role as direct defences against herbivores in solanaceous plants (Ryan, 1989; Haq *et al.*, 2004). Many studies have indicated that JA signalling plays a major role in regulating the levels of TPIs (Koiwa *et al.*, 1997; Paschold *et al.*, 2007; L Wang *et al.*, 2007). Despite the unaltered JA and JA–Ile levels in *NaMKK1*-silenced plants after wounding and simulated herbivory, NaTPI activity was reduced by 50%. Similar inconsistency between JA–Ile and NaTPI activity levels were also seen in NaMEK2-VIGS plants: after wounding, NaMEK2-VIGS plants did not have lower levels of NaTPI activity than did EV plants, although wounding-induced JA–Ile levels were

reduced. These results reveal a complex regulatory network, including MAPK cascades (more specifically, certain transcription factors that are probably controlled directly by MAPK cascades) and JA signalling, in modulating TPI defence in responses to wounding and herbivory. Probably due to the low temperatures required for VIGS, other defence-related secondary metabolites (nicotine, CP, and DTGs) were not elevated after either W+W or W+OS, even in EV plants. Given the critical role of JA signalling in regulating CP and DTGs (Paschold *et al.*, 2007), it is expected that the contents of these compounds in *NaMEK2*-silenced plants are also decreased, and NaMKK1 may also control the levels of these compounds in a largely JA signalling-independent manner. This hypothesis should be tested in plants whose *NaMKK1* and *NaMEK2* are stably silenced with an RNAi approach.

Although NaTPI activity levels were decreased in both NaMKK1-VIGS and NaMEK2-VIGS plants, *M. sexta* gained similar masses on these plants compared with those on EV plants. One possibility is that the decrease of NaTPI activity in these plants was not sufficient to weaken plant defence. Moreover, green leave volatiles (GLVs) are released from wounded leaves during insect feeding and these C6 compounds are thought to function as indirect defence, but also feeding stimulants or herbivore attractants (Meldau *et al.*, 2009; Allmann and Baldwin., 2010; Dicke and Baldwin, 2010). In *N. attenuata*, GLVs stimulate *M. sexta* feeding, and silencing *NaSIPK* and *NaWIPK* impairs GLV emission and results in similar larval growth to those fed on wild-type plants, despite their decreased contents of direct defensive compounds (Meldau *et al.*, 2009). This might also account for the normal growth of *M. sexta* on NaMEK2-VIGS plants. Whether NaMKK1 also controls GLV emission requires further investigation.

In *Arabidopsis* and rice, two close homologues of *MEK2* exist (*AtMKK4* and *AtMKK5* in *Arabidopsis* and *OsMKK4* and *OsMKK5* in rice). However, only one *MEK2* was found in the EST database of *N. tabacum* and *N. benthamiana* and in an *N. attenuata* transcriptome database prepared by 454 sequencing. It is possible that one of the two paralogues of *MEK2* was deleted from the genomes of *Nicotiana* spp. or it is not expressed. More sequence information and phylogeny analyses will provide valuable information about the evolution of *MEK2*—whether *MEK2* is an ancient *MAPKK* that appeared before the divergence of monocots and dicots or the gene duplication of *MEK2* in *Arabidopsis* and rice is completely independent. Assuming that another copy of *MEK2* does exist in *Nicotiana* spp., perhaps it is only expressed in specific organs or tissues. Alternatively, its transcript levels might be very low, and, if so, the possibility of co-silencing by the pTV-NaMEK2 construct cannot be ruled out, given the high sequence similarity between AtMKK4 and AtMKK5. A similar scenario may also apply to *NaMKK1*, which could also have one or more paralogues in *N. attenuata*, although current data point to the likelihood that *NaMEK2* and *NaMKK1* are single-copy genes.

Taken together, the present analyses indicate the involvement of two MAPKKs, NaMKK1 and NaMEK2, in plant responses to herbivores. Gene silencing revealed that these MAPKKs are upstream of different MAPKs and play overlapping but distinct roles in wounding- and herbivory-induced defence. Identification of other important components in wounding- and herbivory-specific signalling pathways, such as MAPKKKs, MAPKKs, MAPKs, and transcription factors, will greatly facilitate our understanding of how plants have evolved to cope with these stresses. Field studies will further reveal the ecological significance of these regulators in plant interactions with various herbivore feeding guilds.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Alignment of the protein sequences of *N. attenuata* NaMKK1 and NaMEK2 with *N. benthamiana* NbMKK1 and *N. tabacum* NtMEK2.

Figure S2 Wounding- and simulated herbivory-induced MAPK activity in EV, NaMKK1-VIGS, and NaMEK2-VIGS plants.

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Table S4. Primers used for qPCR.

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Supplementary Material

Supplementary Table S1. Primers used to clone the open reading frames of *NaMKK1* and *NaMEK2* in *N. attenuata*

Supplementary Table S2. Primers used to clone partial *NaMKK1* and *NaMEK2* into pTV00 to obtain VIGS constructs

Supplementary Table S3. GenBank accession numbers or Swiss-Prot accession numbers of the MAPKKs for phylogenetic analysis

Supplementary Table S4. Primers used for qPCR

Supplementary Fig. S1. Alignment of the protein sequences of *N. attenuata* NaMKK1 and NaMEK2 with *N. benthamiana* NbMKK1 and *N. tabacum* NtMEK2.

Supplementary Fig. S2 Wounding- and simulated herbivory-induced MAPK activity in EV, MKK1-VIGS, and MEK2-VIGS plants.

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Supplementary Table S1 Primers used to clone the open reading frames of *NaMKK1* and *NaMEK2* in *N. attenuata*

Primer	Sequence (5'-3')
NaMKK1 ORF 1	ACCCGACCCTGTTTACCCATC
NaMKK1 ORF 2	AATTAAGCTTGCACGAACTGTC
NaMEK2 ORF 1	CAATCAATCAATCATGCGACC
NaMEK2 ORF 2	CCCATCATCCCCATTAATAGA

Supplementary Table S2 Primers used to clone partial *NaMKK1* and *NaMEK2* into pTV00 to obtain VIGS constructs

Primer	Sequence (5'-3')
NaMKK1-VIGS-BamHI	CTGA**GGATCC**GCAGCGGATATTTGGAGT
NaMKK1-VIGS-HindIII	GTCA**AAGCTT**CTGTAAACAACACTCGATAA
NaMEK2-VIGS-HindIII	GTCA**AAGCTT**CCTCGTGGTTACCGTAGA
NaMEK2-VIGS-ClaI	ATCG**ATCGAT**CTTCCGCCCCTTCTTCCT

Note: bold and underlined nucleotides are restriction sites for cloning into pTV00 vector

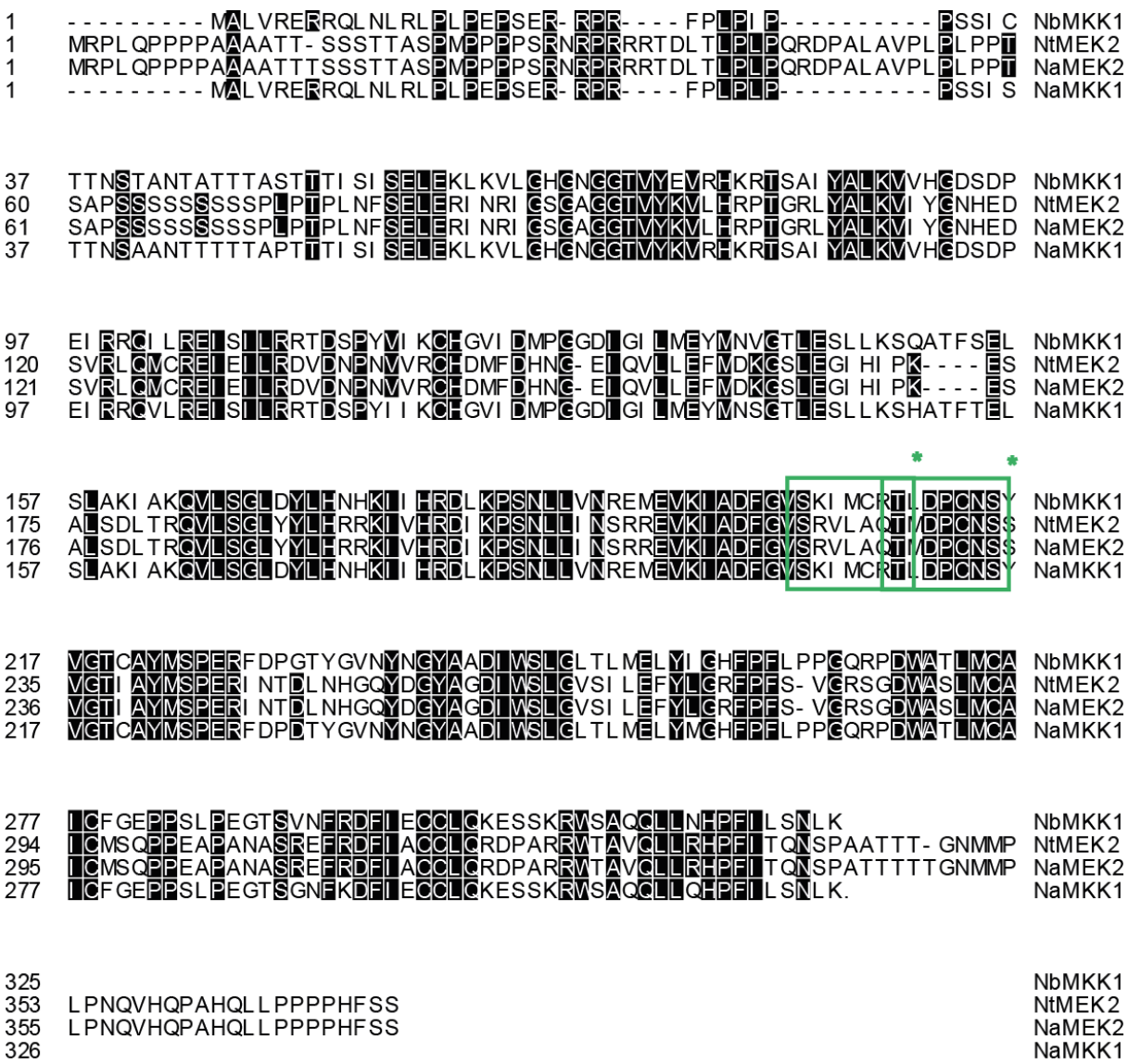
Supplementary Table S3 GenBank accession numbers or Swiss-Prot accession numbers of the MAPKKs for phylogenetic analysis

Gene name	Accession number	Gene name	Accession number
NaMEK2	HQ023234	OsMKK1	EF5296231
NaMKK1	HQ023235	OsMEK2	Os06g05520
AtMKK1	At4g26070	OsMKK3	Os06g27890
AtMKK2	At4g29810	OsMKK4	Os02g54600
AtMKK3	At5g40440	OsMKK5	Os06g09180
AtMKK4	At1g51660	OsMEK1	Q5QN75.1
AtMKK5	At3g21220	NtMEK2	BAE97401
AtMKK6	At5g56580	NtSIPKK	AAF67262
AtMKK7	At1g18350	NtNPK2	BAA06731
AtMKK8	At3g06230	NbMKK1	AB243987
AtMKK9	At1g73500	NbMEK2	AB360636
AtMKK10	At1g32320		

Note: the species of origin of the MAPKKs are indicated by the two letters in front of the protein names; At, *Arabidopsis thaliana*; Na, *Nicotiana attenuata*; Nb, *Nicotiana benthamiana*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*.

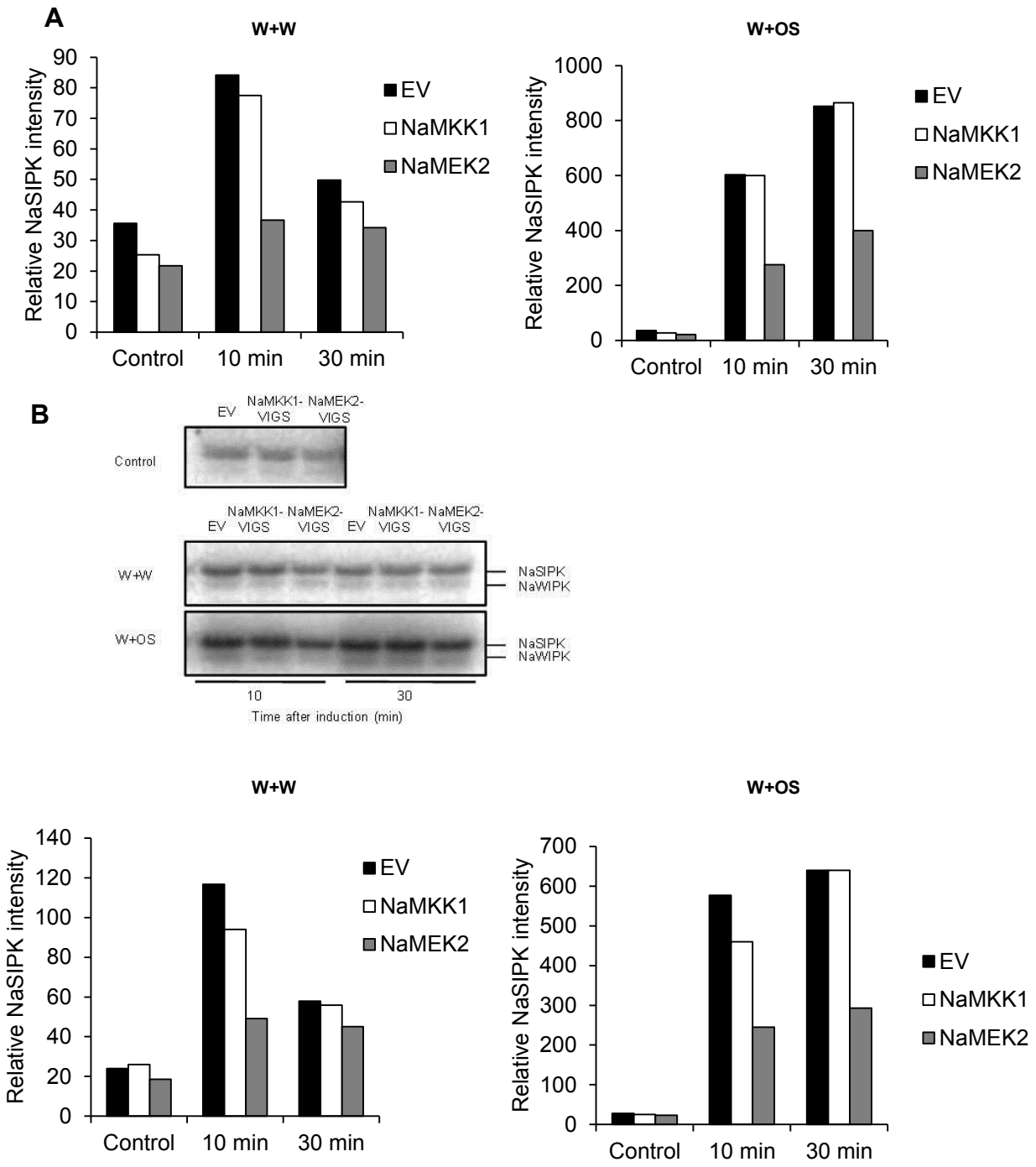
Supplementary Table S4 Primers used for qPCR

Primer	Sequence (5'-3')
NaMKK1-Forward	ATC CCG AGA TTC GCC GTC AA
NaMKK1-Reverse	TCG AGT GTG CCG GAG TTC AT
NaMEK2-Forward	GAG AGT GCT GGC ACA AAC TAT
NaMEK2-Reverse	TCC ATA TAT CTC CAG CAT ACC C
NaEF1a-Forward	CCACACTTCCCACATTGCTG
NaEF1a-Reverse	CGCATGTCCCTCACAGCAAA



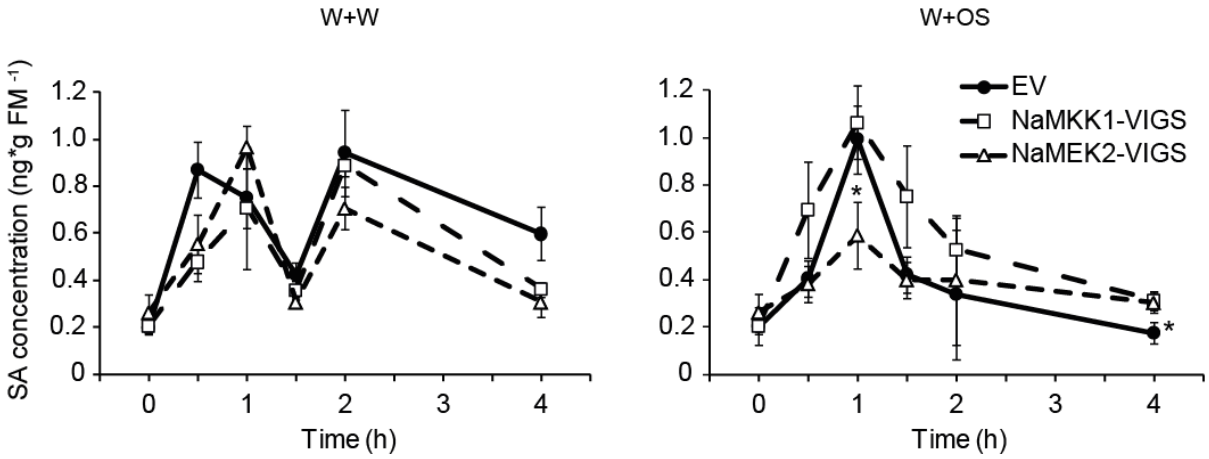
Supplementary Fig. S1 Alignment of the protein sequences of *N. attenuata* NaMKK1 and NaMEK2 with *N. benthamiana* NbMKK1 and *N. tabacum* NtMEK2.

Protein sequences of NaMKK1, NaMEK2, NbMKK1, and NtMEK2 were aligned using ClustalW algorithm. Shaded amino acid sequences represent residues that match the consensus sequence. The conserved motif of plant MAPKKs [S/TxxxxxS/T] are highlighted with green frames (two consecutive motives).



Supplementary Fig. S2 Wounding- and simulated herbivory-induced MAPK activity in EV, MKK1-VIGS, and MEK2-VIGS plants.

N. attenuata plants were wounded with a fabric pattern wheel and 15 µl of water or *M. sexta* oral secretions (OS) were applied immediately to wounds (W+W and W+OS respectively); untreated plants served as controls. In-gel kinase activity assays were performed to determine the activity of NaSIPK and NaWIPK. (A) The band intensities of NaSIPK in Fig. 3B. (B) In-gel MAPK activity assay (upper panel) and NaSIPK band intensities (lower panel) obtained from an independently repeated experiment.



Supplementary Fig. S3 Salicylic acid accumulation in EV, MKK1-VIGS and MEK2-VIGS plants after W+W and W+OS treatment.

Rosette leaves of EV, MKK1-VIGS, and MEK2-VIGS plants were wounded with a fabric pattern wheel and 15µl water or *M. sexta* oral secretions (OS) were applied to wounds. Samples were harvested after indicated times and SA levels were analyzed with LC/MS method. Asterisks indicate significant differences between EV and NaMKK1-VIGS or NaMEK2-VIGS plants (*t*-test; *, $P < 0.05$; $N = 5$).

Chapter 4:

Three MAPK Kinases, MEK1, SIPKK, and NPK2, are not Involved in Activation of SIPK after Wounding and Herbivore Feeding but Important for Accumulation of Trypsin Proteinase Inhibitors

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Three MAPK Kinases, MEK1, SIPKK, and NPK2, are not Involved in Activation of SIPK after Wounding and Herbivore Feeding but Important for Accumulation of Trypsin Proteinase Inhibitors

Maria Heinrich · Ian T. Baldwin · Jianqiang Wu

Abstract We identified three *Nicotiana attenuata* mitogen-activated protein kinase (MAPK) kinases (MAPKKs), NaMEK1, NaSIPKK, and NaNPK2, whose transcript levels were up-regulated in a wild tobacco plant, *N. attenuata*, after wounding and simulated herbivore attack. A virus-induced gene silencing approach was used to analyze the functions of these three MAPKKs in plant defense against the tobacco hornworm *Manduca sexta*. NaMEK1 and NaSIPKK influenced the accumulation of the precursor of jasmonic acid (JA), 12-oxo-phytodienoic acid, and silencing *NaSIPKK* enhanced the levels of wounding- and herbivory-induced JA. In-gel kinase assays indicated that all three MAPKKs were not required for the activation of NaSIPK, an important MAPK in plant responses to wounding and herbivory. However, NaMEK1, NaSIPKK, and NaNPK2 appeared to regulate the levels of trypsin proteinase inhibitor activity. Bioassays revealed that *M. sexta* larval growth was not impaired on *N. attenuata* plants silenced in *NaMEK1*, *NaSIPKK*, or *NaNPK2* expression. Our findings suggest that NaSIPKK is involved in JA biosynthesis after herbivore attack without activating NaSIPK, suggesting that the network of MAPK signaling in *N. attenuata*'s defense responses against herbivore attack is more complicated than previously thought.

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M. Heinrich · I. T. Baldwin · J. Wu (✉)
Department of Molecular Ecology,
Max Planck Institute for Chemical Ecology,
Hans-Knöll-Str. 8,
07745 Jena, Germany
e-mail: jwu@ice.mpg.de

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Abbreviations

FACs	Fatty acid–amino acid conjugates
JA	Jasmonic acid
MAPKK	Mitogen-activated protein kinase kinase
OPDA	12-oxo-phytodienoic acid
SIPK	Salicylic acid-induced protein kinase
TPI	Trypsin proteinase inhibitor
VIGS	Virus-induced gene silencing
WIPK	Wound-induced protein kinase

Introduction

Plants activate sophisticated signaling networks to defend themselves against various biotic and abiotic stresses. Insects are one of the major biotic threats for plants; however, plants have also evolved to have complex defense responses against insect attack. Among the defense-related signaling networks, phytohormone signaling pathways play crucial roles in mediating environmental stress responses.

Jasmonic acid (JA) has been intensively studied for its role in mediating antiherbivore defense responses (Howe and Jander 2008; Wasternack 2007; Wu and Baldwin 2010). After being released from cell membranes when plants perceive wounding or herbivore attack, linolenic acid is modified by lipoxygenase (Vick and Zimmerman 1984), allene oxide synthase, and allene oxide synthase, to be converted into 12-oxo-phytodienoic acid (OPDA) (Vick and Zimmermann 1979). After reduction by OPDA

reductase 3 (OPR3) and three further oxidation reactions, JA is formed in peroxisomes (Schaller et al. 2004; Stintzi et al. 2001; Vick and Zimmerman 1984).

The wild tobacco plant, *Nicotiana attenuata*, is attacked by the larvae of *Manduca sexta* in its natural environment, and the resulting feeding damages have a strong impact on plant fitness. To counteract *M. sexta* attack, *N. attenuata* uses a JA burst, which is induced by specific elicitor compounds, fatty acid–amino acid conjugates (FACs), in *M. sexta* oral secretions (OS), to activate herbivory-specific defense responses (Halitschke et al. 2001). JA signaling is required for *N. attenuata* plants to up-regulate the activity of trypsin proteinase inhibitor (TPI) in the leaves (Zavala et al. 2004a) and to emit volatiles that attract the predators of *M. sexta*. These direct and indirect defenses function concurrently to alleviate the negative fitness effects of *M. sexta* attack (Kessler and Baldwin 2001; Paschold et al. 2007).

Mitogen-activated protein kinases (MAPKs) are well conserved in all eukaryotes, and they play a central role in transducing extracellular stimuli to intracellular responses (Romeis 2001; Zhang and Liu 2001). A growing body of literature has highlighted their roles in plant stress responses. In *Arabidopsis thaliana*, several MAPKs and MAPK kinases (MAPKKs), which phosphorylate and thus activate MAPKs, have been identified to be involved in responses to abiotic stresses, such as drought and salt (Cardinale et al. 2002; Kiegerl et al. 2000; Sheen et al. 2000; Xiong and Yang 2003) and biotic stresses such as viral, bacterial, and fungal infections (Pedley and Martin 2005; Rodriguez et al. 2010).

In *N. attenuata* two MAPKs, NaSIPK (salicylic acid-induced protein kinase) and NaWIPK (wound-induced protein kinase) play central roles in plant defenses against herbivore attack (Wu et al. 2007). After *M. sexta* feeding, *N. attenuata* deficient in NaSIPK or NaWIPK accumulate about 10% of WT levels of OPDA (Kallenbach et al. 2010) and also have strongly decreased JA levels (Meldau et al. 2009). Recently, it was found that after herbivore attack, a MAPK kinase (NaMEK2) regulates the activation of NaSIPK (Heinrich et al. 2011). However, silencing of *NaMEK2* did not show an effect on *M. sexta* larval growth, and NaMEK2 only accounts for about 50% of herbivore attack-induced NaSIPK activity. Therefore, we sought to identify additional MAPKKs that might function in defense responses against herbivores. Using a virus-induced gene silencing (VIGS) approach, we show that three MAPKKs, NaMEK1, NaSIPKK, and NaNPK2, are not important for the activation of NaSIPK and NaWIPK after wounding or herbivore attack, but that NaMEK1 and NaSIPKK play a role in influencing the accumulation of JA and its precursor OPDA. Furthermore, these MAPKKs are involved in regulating TPI activity.

Material and Methods

Molecular Cloning and VIGS

N. attenuata MAPK kinases, NaMEK1, NaSIPKK, and NaNPK2 (GenBank accession numbers: HQ386251, HQ386252, and HQ386250), were amplified using Phusion DNA polymerase (Finnzymes Oy, Espoo, Finland) (primer sequences are listed in Supplementary Table S1), and the purified polymerase chain reaction (PCR) products were cloned into the pJET1.2 vector (Fermentas GmbH, St. Leon-Rot, Germany) and sequenced. Partial *NaMEK1*, *NaNPK2*, and *NaSIPKK* sequences were amplified using plasmids as templates and gene-specific primers (listed in Supplementary Table S2). The PCR products were digested with appropriate restriction endonucleases and were further ligated into pTV00 to obtain the constructs pTV-NaMEK1, pTV-NaNPK2, and pTV-NaSIPKK.

Agrobacterium tumefaciens carrying these constructs was inoculated into *N. attenuata* to obtain gene-silenced plants following a procedure optimized for *N. attenuata* (Saedler and Baldwin 2004). Plants inoculated with *A. tumefaciens* carrying pTV00 (empty vector, or EV) were used for comparisons (EV plants). Plants silenced in *NaPDS* (*phytoene desaturase*) were used to monitor the degree of VIGS, since these plants showed a photobleaching phenotype (Saedler and Baldwin 2004). About 14 days after inoculation, when the leaves of *NaPDS*-silenced plants were completely white, experiments were performed.

Phylogenetic Analysis of MAPKKs

MAPKK protein sequences were deduced from their respective nucleotide sequences, which were retrieved from GenBank (accession numbers are listed in Supplementary Table S3). Protein sequences were aligned using the Clustal W algorithm (DNAStar Inc., Madison, WI). Phylogenetic tree and neighbor-joining bootstrap analysis was conducted using MEGA 4 software (Tamura et al. 2007).

Plant Growth and Treatments

In all experiments, plants of the 31st generation of *N. attenuata* inbred line were used. Plants were grown at 22°C under 16 h of light in a growth chamber. In all the experiments, leaves of rosette stage (about 4 to 5 weeks old) plants were used. Wounding was performed by rolling a fabric pattern wheel three times on each side of the midvein. The wounded leaves were immediately supplied with either 15 µl of water (W + W) or 15 µl of 1:5 diluted OS (W + OS) from *M. sexta*. Larvae of *M. sexta* were reared on *N. attenuata* wild-type plants to collect OS until the third to fifth instar as described in Roda et al. (2004).

Transcriptional Analysis

Total RNA was extracted from leaves using the TRIzol reagent (Invitrogen, Paisley, UK). Total RNA (0.5 µg) of each sample was reverse transcribed using oligo(dT)$_{12–18}$ and Superscript II reverse transcriptase (Invitrogen) following manufacturer's instructions. Using qPCR Core kits (Eurogentec, Liege, Belgium), quantitative real-time PCR (qPCR) was carried out on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). To normalize total cDNA concentration variations, the transcript level of elongation factor 1A (*NaEF1A*) was used. The sequences of primers used for qPCR are provided in Supplementary Table S3.

Phytohormone Analysis

Frozen plant material (100 mg) was homogenized in 2-ml microcentrifuge tubes containing two metal balls and 1 ml of ethyl acetate spiked with 200 ng of D_2-JA and $^{13}C_6$-JA-Ile. Homogenization was done twice with 200 strokes/min for 1 min using a Geno/Grinder 2000 (SPEX CertiPrep, Metuchen, NJ). Samples were centrifuged at 13,000*g* for 20 min at 4°C. Using a vacuum concentrator (Eppendorf AG, Hamburg, Germany), the supernatants were dried. The residues were resuspended in 500 µl of 70% methanol by vortexing for 5 min and centrifuged 10 min at 4°C (13,000 *g*). Supernatants were transferred to crimp vials, and sample measurements were carried out as described in Wu et al. (2007). Ethylene emissions were measured on a photoacoustic spectrometer (INVIVO GmbH, Sankt Augustin, Germany) as described in von Dahl and Baldwin (2007). Three leaves of *N. attenuata* plants were treated with W + OS or left untreated for control. Leaves were weighed immediately after treatments and enclosed in a 3-neck 250-ml round-bottom glass flask for 5 h, and then the concentration of collected ethylene was measured.

Analysis of TPI Activity

Trypsin proteinase inhibitor (NaTPI) activity was quantified using a radial diffusion assay protocol described by van Dam et al. (2001).

Protein Extraction and in-gel Kinase Activity Assay

The tissue of five biological replicates was ground in liquid nitrogen and pooled. About 100 mg of tissue was resuspended in 300 µl of extraction buffer (100 mM Hepes pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, one proteinase inhibitor cocktail tablet per 10 ml extraction buffer [Roche, Mannheim, Germany]). Samples were centrifuged at 4°C, 13,000*g*, for 20 min, and the supernatants were transferred to fresh tubes. Protein concentrations were measured using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA) with BSA (Sigma-Aldrich, Hamburg, Germany) as a standard. Ten micrograms of total protein from each sample was used for in-gel kinase activity assay according to a procedure described by Zhang and Klessig (1997). The image of in-gel kinase activity assays was obtained on a phosphorimager (FLA-3000 phosphor imager system; Fuji Photo Film, Stamford, CT), and the band intensities were quantified using the AIDA software (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

M. sexta Growth Bioassays

M. sexta eggs from an in-house reared population were kept in a growth chamber (Snijders Scientific, Tilburg, the

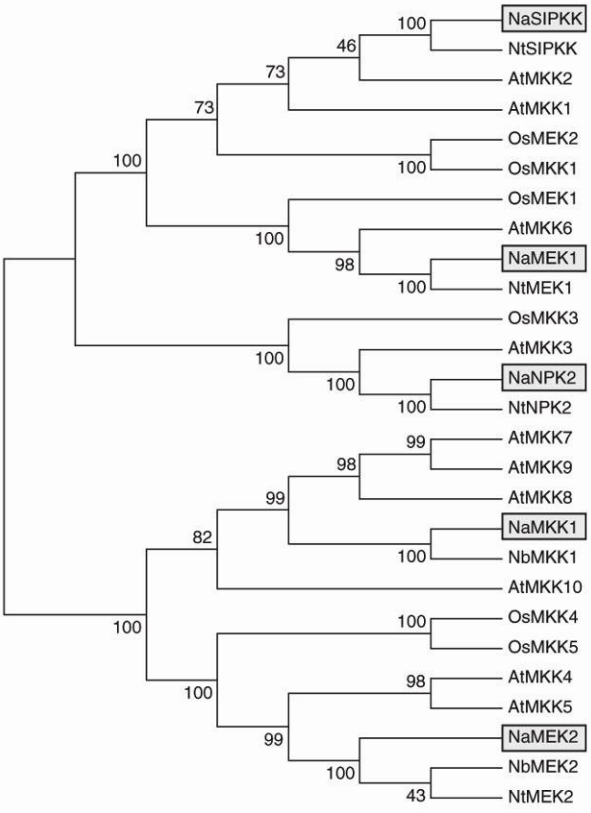

Fig. 1 Phylogenetic analysis of plant mitogen-activated kinase kinases (MAPKKs). Protein sequences of MAPKKs in Arabidopsis, *Nicotiana* spp., and rice were aligned using the Clustal W algorithm. An unrooted neighbor–joining tree and bootstrap analysis were performed with the MEGA 4 program. The *N. attenuata* MAPKKs are highlighted with grey. The species of origin of the MAPKKs are indicated by the abbreviation in front of the protein names: At, *Arabidopsis thaliana*; Na, *Nicotiana attenuata*; Nb, *Nicotiana benthamiana*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*

Netherlands) at 26°C under 16 h of light and at 24°C in 8 h of darkness, until the larvae hatched. Freshly hatched *M. sexta* neonates were placed on fully developed leaves of 30 replicated rosette-stage NaMEK1-VIGS, NaNPK2-VIGS, NaSIPKK-VIGS, and EV plants (one larva per plant). The larval masses were measured on days 5, 9, and 12.

Statistical Analysis

Data were analyzed by unpaired *t* tests using SPSS Statistics Version 17.0 (www.spss.com).

Results

Phylogenetic Analysis of *N. attenuata* NaMEK1, NaSIPKK, and NaNPK2

Using sequences of *NtMEK1*, *NtSIPKK*, and *NtNPK2* from *N. tabacum* (Gomi et al. 2005; Heberle-Bors et al. 2001; Shibata et al. 1995) as references, the open-reading frames of these three MAPKKs in *N. attenuata* were cloned. Phylogenetic analysis (Fig. 1) indicated that *NaMEK1* is a close homologue of *A. thaliana AtMKK6* (Hua et al. 2006), *NaSIPKK* is grouped closely with *A. thaliana AtMKK1* and *AtMKK2* (Xing et al. 2009), and *NaNPK2* is closely related to *AtMKK3* (Hua et al. 2006; Ichimura et al. 1998). Protein sequence alignment of the all these MAPKKs, including NaMEK2 and NaMKK1 (Heinrich et al. 2011), confirmed the existence of the conserved motif sequence [S/TxxxxxS/T] of MAPKKs (Supplementary Fig. S1) (MAPK Group 2002). It is likely that all three MAPKKs are single genes in *N. attenuata*, since searching tobacco EST database and an *N. attenuata* transcriptome database obtained by 454 sequencing revealed no other close homologues, although the possibility that they have paralogues, which might have low expression levels or are expressed in specific organs or tissues, cannot be ruled out. Consistently, Liu et al. (2011) proposed that in high plants, MAPKK6, which is a close homologue of NaMEK1, exists as single copy genes.

Transcriptional Analysis of NaMEK1, NaSIPKK, and NaNPK2

To elucidate the transcriptional responses of the three MAPKKs in *N. attenuata* after wounding and mimicked herbivore attack, leaves of rosette-stage plants were wounded with a fabric pattern wheel and supplied with either 15 µl

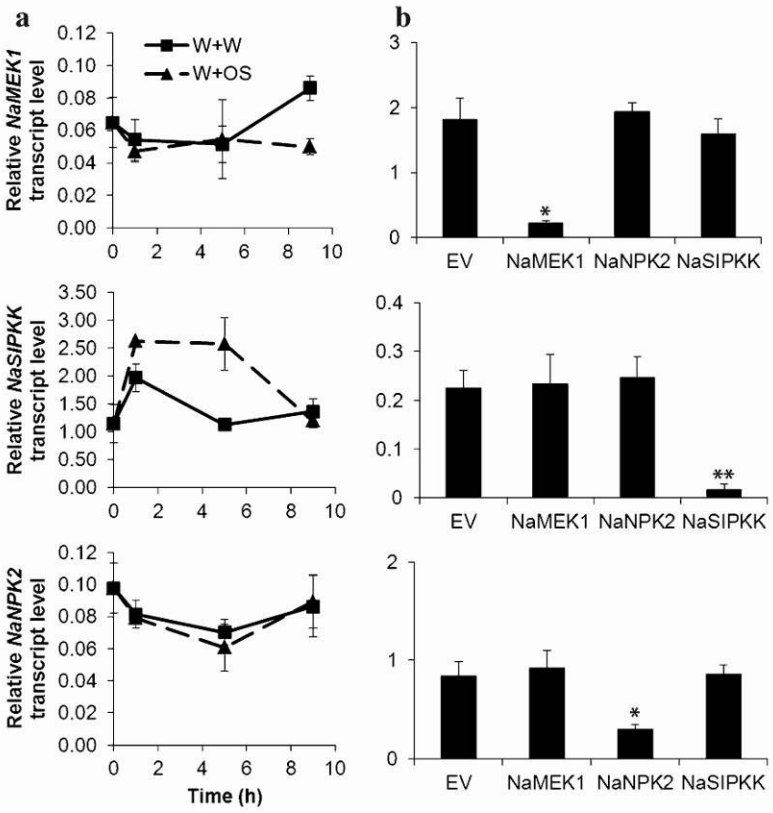

Fig. 2 Transcript regulation of *NaMEK1*, *NaSIPKK* and *NaNPK2* in wild-type *N. attenuata* plants challenged with wounding and simulated herbivory and silencing of these genes using VIGS. **a** Transcript levels of *NaMEK1*, *NaSIPKK* and *NaNPK2* after wounding and simulated herbivory. Plants were wounded with a fabric pattern wheel, and 15 µL of water or *M. sexta* oral secretions (OS) were applied immediately to wounds (W + W and W + OS, respectively); untreated plants served as controls. Samples were harvested after 1, 5, and 9 h and the transcript levels were analyzed by qPCR. **b** Analysis of the silencing efficiency and specificity *NaMEK1*, *NaSIPKK*, and *NaNPK2* in NaMEK1-, NaSIPKK- and NaNPK2-VIGS. Leaf tissue remained untreated, empty vector (EV) plants served as control. Asterisks indicate significant differences between EV and NaMEK1-VIGS, NaSIPKK-VIGS or NaNPK2-VIGS plants (mean ± SE; t-test; $*P < 0.05$; $**P < 0.01$; $n=5$)

water (W + W) or 15 µl 5-times diluted *M. sexta* OS (W + OS). qPCR showed 50% higher expression of *NaMEK1* 9 h after W + W treatment compared with W + OS (Fig. 2a). Compared with W + W, W + OS treatment induced 50% greater transcript levels of *NaSIPKK* 1 h after treatments and W + OS-induced *NaSIPKK* transcript levels did not decreased by 5 h, while those induced by W + W dropped to the basal levels. *NaNPK2* transcript levels slowly decreased after W + W and W + OS treatment, and no different levels were found in samples treated with W + W and W + OS (Fig. 2a). To analyze the function of these MAPKKs, a transient silencing method, VIGS, was used. *A. tumefaciens* cells carrying pTV00, pTV-NaMEK1, pTV-NaSIPKK, and pTV-NaNPK2 were inoculated into wild-type *N. attenuata* plants to obtain EV, NaMEK1-VIGS, NaSIPKK-VIGS, and NaNPK2-VIGS plants. qPCR analysis indicated specific silencing of *NaMEK1*, *NaSIPKK*, and *NaNPK2* gene expression in these plants (Fig. 2b). None of these plants showed an abnormal growth phenotype (Supplementary Fig. 2).

NaMEK1 And NaSIPKK are Involved in Regulating the Accumulation of OPDA and JA

JA plays a key role in regulating herbivore defense responses (Creelman and Mullet 1997; Howe 2011; Howe and Jander 2008); thus, we determined its contents in EV, NaMEK1-VIGS, NaSIPKK-VIGS, and NaNPK2-VIGS plants 1 h after treatment with W + W and W + OS (Fig. 3a). Although the JA contents in NaNPK2-VIGS plants were slightly higher than in other plants when uninduced, after inductions, NaNPK2-VIGS showed similar levels of JA to those in EV (Fig. 3a). After the 1-h W + OS treatment, EV plants accumulated about 4,400 ng/g fresh mass (FM), while NaMEK1-VIGS plants had about 1,000 ng/g FM more JA; no difference in JA levels were found between W + W-induced EV and NaMEK1-VIGS plants (Fig. 3a). The highest JA amount was detected in NaSIPKK-VIGS plants 1 h after W + OS, which reached a level of 7,000 ng/g FM; consistently, 74% greater JA was also found in W + W-treated NaSIPKK-VIGS than in EV.

OPDA is a precursor of the JA, whose levels are rapidly elevated but then quickly decline after W + OS (Kallenbach et al. 2010). Therefore, samples were taken from plants that had been treated with W + OS for 5 and 10 min. Although NaSIPKK-VIGS plants had greater JA levels than did EV, lower OPDA contents in NaSIPKK-VIGS plants were found. NaMEK1-VIGS plants showed similarly reduced OPDA levels. NaNPK2-VIGS did not show any differences in OPDA levels compared with EV (Fig. 3b). These data suggest that NaMEK1 and NaSIPKK

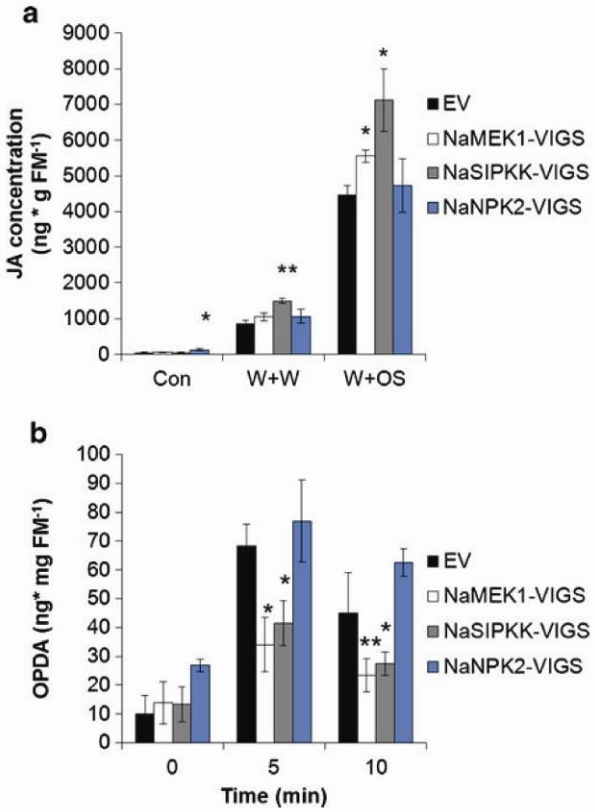

Fig. 3 W + OS-induced JA and OPDA levels in EV, NaMEK1-VIGS, NaSIPKK-VIGS and NaNPK2-VIGS plants. Plants were wounded with a fabric pattern wheel, and 15 µL of water or *M. sexta* oral secretions (OS) were applied immediately to wounds (W + W and W + OS, respectively). **a**) JA contents in control (Con) plants and in plants 1 h after being treated with W + OS. **b** Contents of OPDA 5 and 10 min after W + OS treatment. Asterisks indicate significant differences between EV and NaMEK1-VIGS, NaSIPKK-VIGS or NaNPK2-VIGS plants (mean ± SE; t-test; **P* <0.05; ***P* <0.01; *n*=5)

have an important function in controlling W + OS-induced OPDA accumulation.

NaSIPK Activation and Ethylene Emission are Independent of NaMEK1, NaSIPKK, and NaNPK2

The two MAPKs, NaSIPK and NaWIPK, are required for the herbivory-induced JA burst in *N. attenuata*. Our previous work indicated that NaMEK2 is required for the activation of NaSIPK and NaWIPK (Heinrich et al. 2011). Therefore, we performed an in-gel kinase assay to analyze whether NaSIPK and NaWIPK are also regulated by these three MAPKKs. Ten minutes after the W + W treatment, EV plants exhibited highly increased NaSIPK activity, and even higher levels were detected after the W + OS treatment (Fig. 4). Thirty minutes after the W + OS treatment, NaSIPK activity in EV plants retained similar levels, while the activity levels of NaSIPK in the W + W-treated samples diminished. In

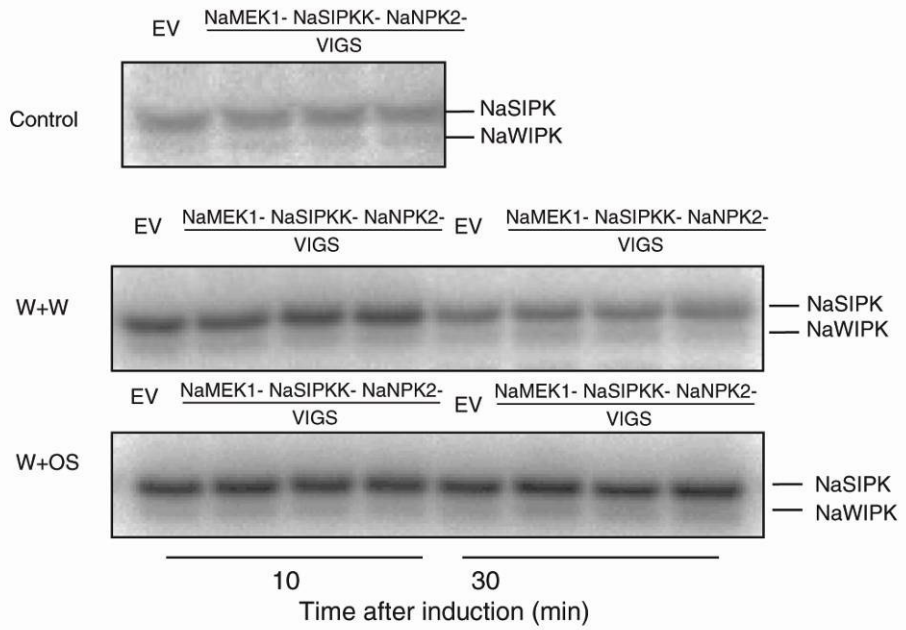

Fig. 4 MAPK activity in wounding- or simulated herbivory-induced NaMEK1-VIGS, NaSIPKK-VIGS and NaNPK2-VIGS plants

contrast to the altered JA levels, NaSIPK activity was not changed in NaMEK1-VIGS, NaSIPKK-VIGS, or NaNPK2-VIGS plants, compared with that in EV (Fig. 4). Whether the activity of NaWIPK was altered in NaMEK1-VIGS, NaSIPKK-VIGS, and NaNPK2-VIGS was not clear, since the band intensities of NaWIPK were too weak.

Ethylene emission is known to be regulated by NaSIPK, whose activity is partly dependent on NaMEK2 (Heinrich et al. 2011; Wu et al. 2007). To investigate if these three MAPKKs have an effect on herbivory-induced ethylene emission, three leaves of EV, NaMEK1-VIGS, NaSIPKK-VIGS, and NaNPK2-VIGS rosette plants were treated with W + OS, and ethylene emissions were quantified. We did not detect any differences in the amount of ethylene among all plants (Fig. 5), which is consistent with the unaltered NaSIPK activity.

NaMEK1, NaSIPKK, and NaNPK2 Influence Herbivory-Induced TPI Accumulation, but not the Larval Growth of *M. sexta*

TPIs are produced in *N. attenuata* after *M. sexta* attack, which slow larval growth by inhibiting the activity of trypsin-like proteases in *M. sexta* larval midguts (Baldwin et al. 2003; van Dam et al. 2001). We treated EV, NaMEK1-VIGS, NaSIPKK-VIGS, and NaNPK2-VIGS plants with W + W and W + OS and collected the samples after 3 days. In the untreated control samples, all three MAPKK-silenced *N. attenuata* plants showed about onefold to threefold elevated levels of NaTPI activity compared with EV control (Fig. 6a). However, 3 days after the W + W treatment, NaMEK1-VIGS and NaSIPKK-VIGS plants exhibited 70% and 50% reduced NaTPI activity levels compared with EV, respectively. Similarly, NaTPI activity levels in the W + OS-treated NaNPK2-VIGS plants were about 60% lower, but the activity of TPI in NaMEK1-VIGS and NaSIPKK-VIGS was not different from that in EV (Fig. 6a). We also examined the contents of other herbivore resistance-related secondary metabolites, nicotine, and chlorogenic acid (Kaur et al. 2010; Steppuhn et al. 2004). No significant changes were detected in these plants after being treated with W + OS (Supplementary Fig. 3).

To examine whether silencing these MAPKKs altered plant defense levels, the larval growth of *M. sexta* on NaMEK1-VIGS, NaSIPKK-VIGS, and NaNPK2-VIGS

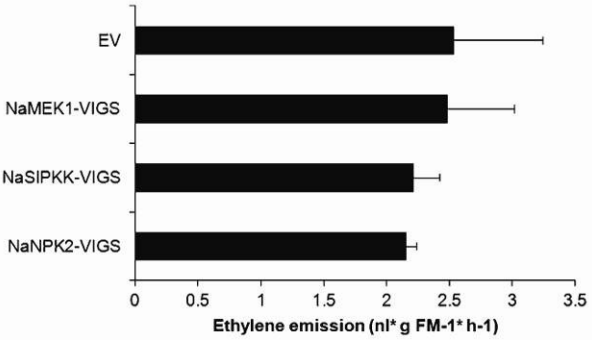

Fig. 5 Ethylene emissions (mean ± SE) from W + OS-treated MAPKK-silenced plants. Ethylene amounts were measured in 3 rosette leaves from each replicated plant which were treated with W + OS treatment

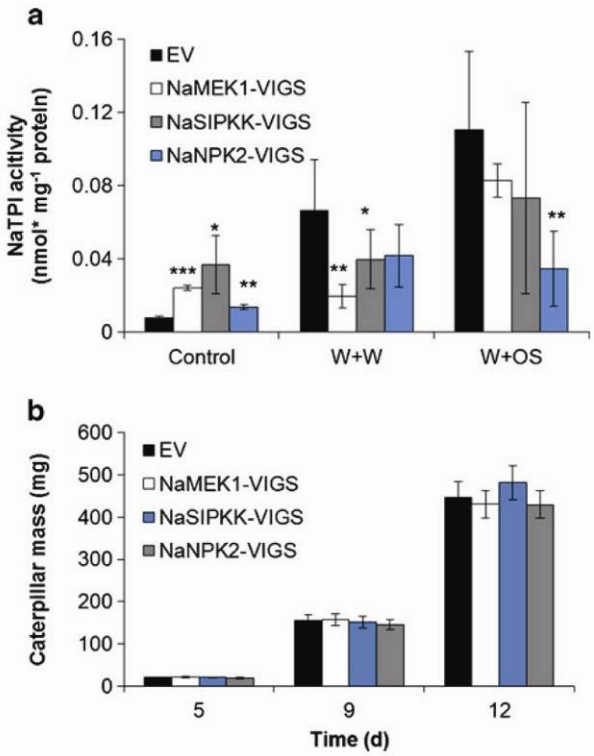

Fig. 6 NaNPK2-VIGS plants have decreased NaTPI activity, but do not exhibit compromised resistance to *M. sexta*. **a** NaTPI activity in EV, NaMEK1-VIGS, NaSIPKK and NaNPK2-VIGS plants. Plants were wounded with a fabric pattern wheel, and 15 μL of water or *M. sexta* oral secretions (OS) were applied immediately to wounds (W + W and W + OS, respectively); untreated plants served as controls. Three days after treatments, samples were collected and NaTPI activity was analyzed. Asterisks indicate significant differences between EV and NaMEK1-VIGS, NaSIPKK-VIGS and NaNPK2-VIGS plants (mean ± SE; t-test; $*P < 0.05$; $**P < 0.01$; $n=5$). **b** *Manduca sexta* mass gain on EV, NaMEK1-VIGS, NaSIPKK-VIGS and NaNPK2-VIGS plants. Each type of plant was infested with 30 *M. sexta* neonates (one larva per plant); larval masses (mean ± SE) were measured after 5, 9, and 12 d

was recorded: no differences in mass gain were found among all plants (Fig. 6b).

Discussion

MAPKs are involved in plant responses to biotic and abiotic stress factors. MAPKs are directly regulated by MAPKKs, which constitute part of the complex regulation network (Asai et al. 2002; Yang et al. 2001). The *A. thaliana* genome contains 60 MAPKKKs, 10 MAPKKS, and 20 MAPKs (MAPK Group 2002), and many of these kinases have been studied for their functions in development and stress responses (Bergmann et al. 2004; Cardinale et al. 2002; Kiegerl et al. 2000; Kovtun et al. 1998; Lukowitz et al. 2004; Rodriguez-Saona et al. 2010; Wang et al. 2007). In *N. attenuata,* the function of only two MAPKKs has been studied (Heinrich et al. 2011). Here, we examined the roles of three other MAPKKs in plant defense responses against herbivores using a reverse genetic approach.

NaMEK1 and NaSIPKK Regulate the JA Burst and Control the Accumulation of OPDA

In *N. attenuata*, JA biosynthesis is modulated by many factors (Wu and Baldwin 2010), and NaSIPK and WIPK are involved in the regulation of the early steps of the JA biosynthetic pathway: silencing *NaSIPK* or *NaWIPK* decreases the levels of wounding- and FAC-elicited OPDA and JA (Kallenbach et al. 2010). It has been suggested that OPDA might act as a signaling molecule as direct defense response to herbivory through the up-regulation of the expression of wound-induced genes (Stintzi et al. 2001). Five and 10 min after the W + OS treatment, NaMEK1-VIGS and NaSIPKK-VIGS plants showed about 40%–50% reduced levels of OPDA, but after the W + OS treatment, JA levels were not lower in these two MAPKK-silenced plants than in EV; especially NaSIPKK-VIGS plants even showed higher JA contents (Fig. 3). These findings are consistent with the notion that NaMEK1 and NaSIPKK might play a role in the biosynthesis of OPDA, or they control the conversion rate of OPDA to JA.

It has been shown that NtMPK4 (a MAPK) in *N. tabacum* is activated by NtSIPKK, and NtMPK4 is required for JA signaling and is involved in stomata movement (Gomi et al. 2005). *N. attenuata* plants silenced in *NaSIPKK* showed an enhanced JA accumulation after herbivore attack. Consistently, silencing *NaMPK4*, which is likely the downstream MAPK of NaSIPKK in *N. attenuata*, also leads to greater W + OS-induced JA levels (Hettenhausen, Baldwin, and Wu, in review). Whether NaSIPKK has an effect on stomatal movement or responses to drought and salt still needs to be investigated. MPK4 in *A. thaliana* is required for cytokinesis (Kosetsu et al. 2010), and silencing *AtMKK6* (the homologue of NaMEK1) or *AtMPK4 Arabidopsis* plants show dwarfism (Kosetsu et al. 2010). However, none of these MAPKK-silenced *N. attenuata* plants showed altered morphology compared with EV plants (Supplementary Fig. 2). Therefore, it is likely that NaMEK1 and AtMKK6 are functionally different, although they have somewhat similar sequences. This possibility should be examined in stably silenced plants, whose development can be examined throughout the plant life cycle.

NaMEK1, NaSIPKK, and NaNPK2 are Involved in TPI Regulation in a NaSIPK-Independent Manner

TPIs are important in *N. attenuata*'s defense against *M. sexta* feeding (Zavala et al. 2004b). Basal levels of NaTPI activity

are up-regulated in NaMEK1-VIGS, NaSIPKK-VIGS, and NaNPK2-VIGS plants. However, after the W + W treatment, NaMEK1-VIGS and NaSIPKK-VIGS plants showed decreased NaTPI activity levels. After the W + OS treatment, only NaNPK2-VIGS lines showed significantly lower levels of NaTPI activity (Fig. 6). The change from the higher basal level to a lower NaTPI activity level after wounding suggests the involvement of the MAPKKs in the herbivore-induced up-regulation of NaTPI. In-gel kinase activity assays indicated that NaMEK1, NaSIPKK, and NaNPK2 are not important for the activation of NaSIPK (Fig. 4). Thus, these MAPKKs may not be located upstream of NaSIPK, or they have redundant functions and silencing individual genes specifically does not affect the activation of NaSIPK. Furthermore, besides NaSIPK (Wu et al. 2007), NaTPI is also modulated by other MAPK pathways that involve NaMEK1, NaSIPKK, NaNPK2, as well as two previously studied NaMEK2 and NaMKK1 (Heinrich et al. 2011). JA signaling plays an important role in regulating the activity of TPI (Paschold et al. 2007). Notably, after the W + OS treatment, NaSIPKK-VIGS plants had greater levels of JA than did EV, but TPI activity was lower in these plants than in EV. Therefore, JA signaling is required but not sufficient for controlling TPI activity, and the regulatory network that modulates TPI activity includes at least JA signaling and MAPK cascades containing NaSIPKK, NaMEK1, NaMEK2 NaNPK2, NaMKK1, and MAPKs (such as NaSIPK and NaWIPK) (Wu et al. 2007).

M. sexta larvae did not grow larger on the MAPKK-silenced plants, indicating that silencing these genes did not strongly decrease the defense levels of these plants. This is consistent with the fact that important defense-related metabolites such as nicotine and chlorogenic acid (Kaur et al. 2010; Steppuhn et al. 2004) were not changed in the three MAPKK silenced lines (Supplementary Fig. 3). Either these MAPKKs are not important kinases for herbivore resistance or they are redundant, and compromising one kinase is not sufficient to strongly affect plant secondary metabolites. Although NaTPI is known to be important in defense of *N. attenuata* against *M. sexta*, lower NaTPI levels in NaNPK2-VIGS plants did not promote the larval growth. We suspect that silencing *NaNPK2* may have enhanced the level of an unknown defensive compound, and thus, NaNPK2-VIGS plants did not show changes in total defense level. In *N. attenuata* plants silenced in *NaSIPK* or *NaWIPK*, a decreased TPI activity was also observed; however, these plants emitted lower levels of green leaf volatiles (GLVs), which function as feeding stimulants for *M. sexta*, resulting in decreased feeding of *M. sexta* (Meldau et al. 2009). Thus, it is also possible that NaNPK2 also regulates GLV production, and NaNPK2-VIGS plants had lower GLV emissions. These hypotheses should be examined further.

To investigate the functions of these MAPKKs in plant resistance to environmental stresses, stably transformed lines of MAPKKs should be produced to unravel the complex network of MAPKKs, since the analysis of plants silenced using VIGS is limited to the specific growth conditions, which are required by the VIGs procedure, such as low temperature and high humidity. Additionally, creating silenced lines in which more than one MAPKK is knocked down is important for studying whether these MAPKKs have overlapping functions. It has already been reported that different MAPKKs can regulate the same MAPKs after different stress stimuli. For example, in *Arabidopsis*, AtMPK3 and AtMPK6 are regulated by AtMKK9 in mediating ethylene signaling (Yoo et al. 2008), and they are also phosphorylated after flagellin elicitation by AtMKK4 and AtMKK5 (Asai et al. 2002). In addition, proteomic analysis could shed light on the function of MAPKKs in the interaction between *M. sexta* and *N. attenuata* (Zhang et al. 2010). The fitness consequences of these stably silenced lines should be tested under field conditions in *N. attenuata*'s natural environment, in which plants are challenged with various biotic and abiotic stresses.

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Supplemental material

Supplemental Table S1. Primers used to clone the open reading frames of *NaMEK1, NaSIPKK* and *NaNPK2* in *N. attenuata*

Supplemental Table S2. Primers used to clone partial *NaMEK1, NaSIPKK* and *NaNPK2* into pTV00 to obtain VIGS constructs

Supplemental Table S3. Primers used for qPCR

Supplemental Table S4. GenBank or Swiss-Prot accession numbers of the MAPKKs for phylogenetic analysis

Supplemental Fig. S1. Alignment of the protein sequences of *N. attenuata* NaMEK1, NaSIPKK, and NaNPK2 with *N. attenuata* NaMKK1 and NaMEK2.

Supplemental Fig. S2. Morphology of NaMEK1-VIGS, NaSIPKK-VIGS, NaNPK2-VIGS and EV plant.

Supplemental Fig. S3 Secondary metabolite levels in NaMEK1-VIGS, NaSIPKK-VIGS, NaNPK2-VIGS, and EV plants three days after W+W and W+OS treatment.

Supplemental Table S1 Primer used to clone *N. attenuata* MAPKKs

Primer	Sequence (5‘-3‘)
NaMEK1 ORF 1	ATGAAGACGACGAAGCCATTGA
NaMEK1 ORF 2	TTATCTTGGAAAATTTACAGGAGG
NaNPK2 ORF 1	ATGGCTGGGTTGGAGGAATTGA
NaNPK2 ORF 2	CTATTGAGTAATGAAAAGTTCTTGCT
NaSIPKK 1	CTTTCTCTCCCTCCTCCTGA
NaSIPKK 2	AGGTCCTGCCGATGTGAAGT

Supplemental Table S**2** Primer used to clone MAPKKs into PTV00 to obtain VIGS-constructs

Primer	Sequence (5‘-3‘)
NaMEK1-VIGS-*Bam*HI	CTGA**GGATCC**CCACCACTTCCCTTTCCG
NaMEK1-VIGS-*Hind*III	GTCA**AAGCTT**CTTAAGCTCTCTGTACCTG
NaNPK2-VIGS-*Bam*HI	CTGA**GGATCC**GGAATGTGAATAGCTCTCT
NaNPK2-VIGS-*Hind*III	GTCA**AAGCTT**GATTCATACTCTCTATCCGA
NaSIPKK-VIGS-*Bam*HI	CTGA**GGATCC**CTTGCGCATGGACTCTTC
NaSIPKK-VIGS-*Hind*III	GTCA**AAGCTT**GGAACGTTTAAGGATGGAG

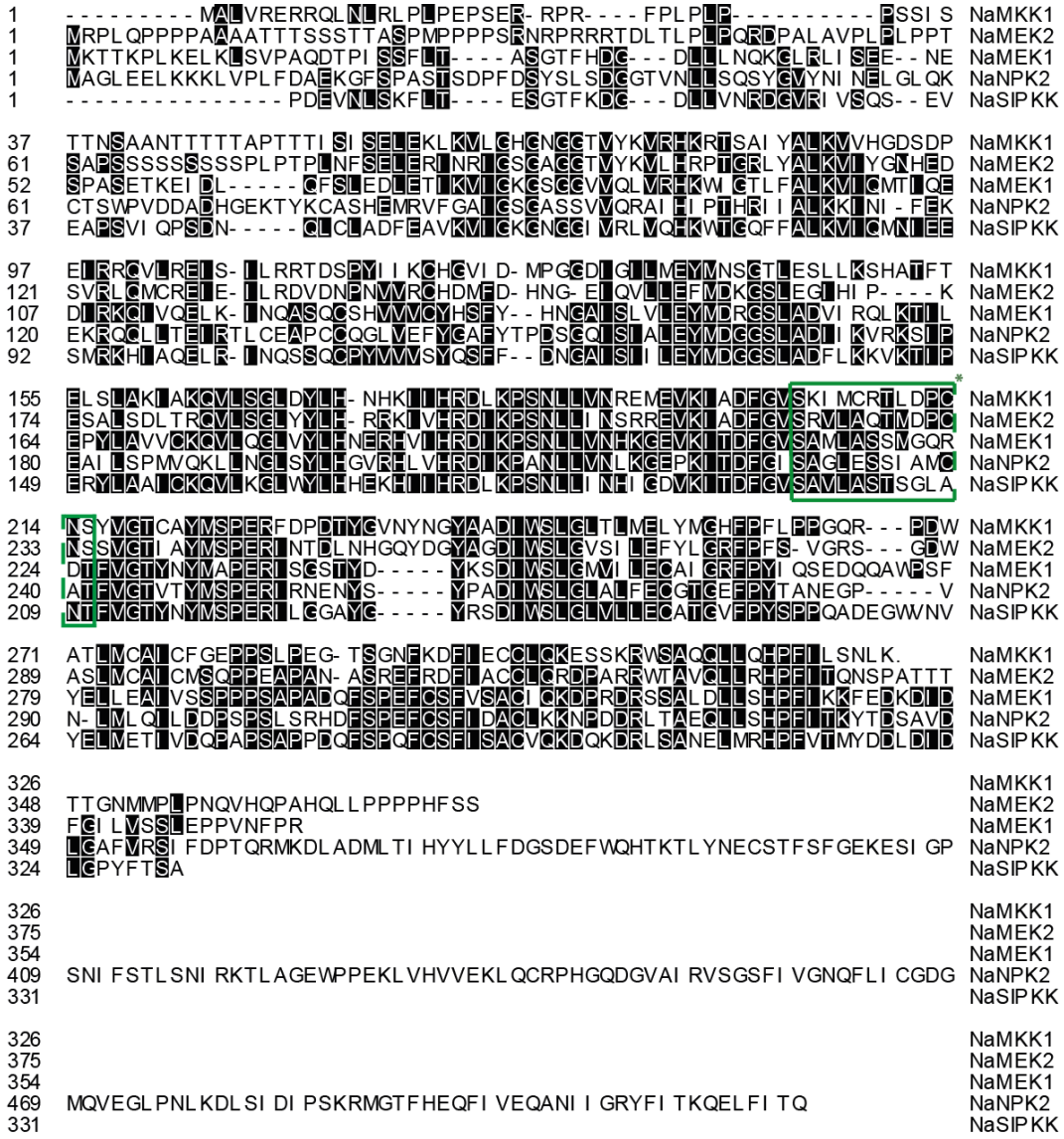
Supplemental Table S3 GenBank accession numbers or Swiss-Prot accession numbers of the MAPKKs for phylogenetic analysis

Gene name	Accession number	Gene name	Accession number
NaMEK2	HQ023234	AtMKK10	At1g32320
NaMKK1	HQ023235	OsMKK1	EF5296231
NaMEK1	HQ386251	OsMEK2	Os06g05520
NaNPK2	HQ386250	OsMKK3	Os06g27890
NaSIPKK	HQ386252	OsMKK4	Os02g54600
AtMKK1	At4g26070	OsMKK5	Os06g09180
AtMKK2	At4g29810	OsMEK1	Q5QN75.1
AtMKK3	At5g40440	NtMEK1	AJ302651
AtMKK4	At1g51660	NtMEK2	BAE97401
AtMKK5	At3g21220	NtSIPKK	AAF67262
AtMKK6	At5g56580	NtNPK2	BAA06731
AtMKK7	At1g18350	NbMKK1	AB243987
AtMKK8	At3g06230	NbMEK2	AB360636
AtMKK9	At1g32320		

Note: the species of origin of the MAPKKs are indicated by the two letters in front of the protein names; At, *Arabidopsis thaliana*; Na, *Nicotiana attenuata*; Nb, *Nicotiana benthamiana*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*.

Supplemental Table S**4** Primer used for qPCR.

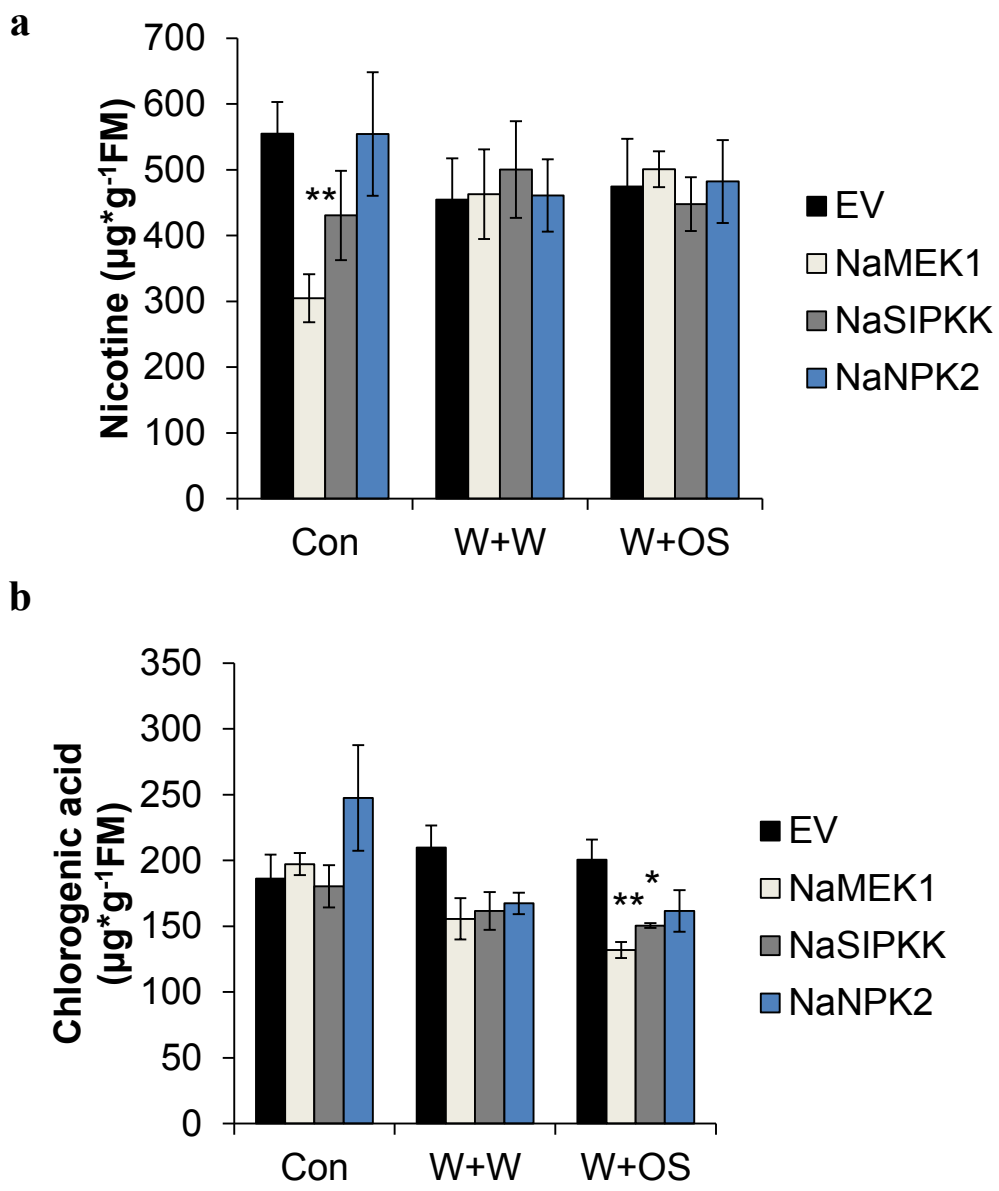
Primer	**Sequence (5‘-3‘)**
NaMEK1 forward	CTA GCC AGC TCT ATG GGT CA
NaMEK1 reverse	CCA AGC TCC AGA TAT CAC TCT
NaSIPKK forward	CGA ACG ATA TCT TGC TGC CAT
NaSIPKK reverse	CAC TCA CAC CAA AGT CAG TGA
NaNPK2 forward	ACC AGA GTT CTG CTC GTT CAT T
NaNPK2 reverse	CAAGTCCACTGCAGAATCAGTA
Elongation factor 1a forward	CCACACTTCCCACATTGCTG
Elongation factor 1a reverse	CGCATGTCCCTCACAGCAAA



Supplemental Figure 1 Alignment of the protein sequences of 5 *N. attenuata* MAPKKs. Protein Alignment was done with MegAlign(DNASTAR Software). Letters with black background are residues that match the consensus sequence. The sequence motif for plant MAPKKs [S/TxxxxxS/T] is highlighted with the green box (MAPK Group et al., 2002). Sequences of NaMEK2 and NaMKK1 have been described in Heinrich *et al* (2011).

NaPDS-VIGS | EV | NaMEK1-VIGS | NaSIPKK-VIGS | NaNPK2-VIGS

Supplemental Figure 2 Plant morphology is not changed in NaMEK1-VIGS, NaSIPKK-VIGS and NaNPK2-VIGS plants. Early rosette-staged plants were inoculated with *Agrobacterium* carrying pTV00, pTV-NaMEK1, pTV-NaNPK2 and pTV-NaSIPKK to form EV, NaMEK1-VIGS, NaNPK2-VIGs, and NaSIPKK-VIGS plants. Photos were taken 21 days after inoculation. NaPDS-VIGS were created by inoculating pTV-NaPDS, which has a photo-bleaching phenotype and indicates the completion of silencing.



Supplemental Figure 3 NaMEK1-VIGS, NaSIPKK-VIGS and NaNPK2-VIGS show minor changes in a) nicotine and b) chlorogenic acid amounts 3 days after W+OS treatment. Asterisks indicate significant differences between EV and NaMEK1-VIGS, NaSIPKK-VIGS or NaNPK2-VIGS plants (t-test; *P <0.05; **P <0.01; n=5).

Chapter 5

Jasmonic acid specifically suppresses the transcript accumulation of *GA20ox* gene and thus inhibits the biosynthesis of gibberellic acid in the stem of irCDPK4/5 plants

Jasmonic acid specifically suppresses the transcript accumulation of *GA20ox* gene and thus inhibits the biosynthesis of gibberellic acid in the stem of irCDPK4/5 plants

Maria Heinrich, Christian Hettenhausen, Theodor Lange, Hendrik Wünsche, Jing-Jing Fang, Ian T. Baldwin, and Jianqiang Wu

Department of Molecular Ecology, Max Planck Institute for Chemical Ecology

Hans-Knöll-Str. 8, D-07745, Jena, Germany

Running title
CDPK4/5 changes the stem morphology of *N. attenuata*

Corresponding author: Jianqiang Wu; Email: jwu@ice.mpg.de

Phone: +49 (0)3641 57 1122

Fax: +49 (0)3641 57 1125
E-mail: jwu@ice.mpg.de

Footnote: The author responsible for distribution of materials integral to the findings presented in this article is: Ian T. Baldwin (baldwin@ice.mpg.de).

Abbreviations

CDPK4/5	calcium-dependent protein kinase 4/5
ir	inverted repeat
GA	gibberellic acid
JA	jasmonic acid
VIGS	virus-induced gene silencing

Abstract

The calcium-dependent protein kinases 4 and 5 (CDPK4/5) have been shown to be involved in herbivore and pathogen defense in the wild tobacco plant *Nicotiana attenuata*. Despite their role in defense responses silencing of CDPK4/5 seems to cause even more severe phenotypical and functional changes. Silenced CDPK4/5 plants show curly leaves, delayed senescence, they lose apical dominance and show a delayed stem growth. The constant level of jasmonic acid and secondary defense metabolites is much higher in the stems of silenced CDPK4/5 plants compared to wild type. Here we describe the phenotypical changes induced by stably silencing CDPK4/5 in *N. attenuata* and the role of these genes in growth and biosynthesis regulation processes. Using the toolbox of crossing we found evidence that the growth phenotype depends on jasmonic acid and gibberellic acid. Giving CDPK4/5 a central role in hormone crosstalk we analyzed gibberellic and jasmonic acid antagonism. Application of gibberellic acid rescues the stem growth of irCDPK4/5 plants to wild type level and expression analysis revealed the differential regulation of several GA biosynthesis genes. Transient gene silencing of an important GA biosynthesis gene, *GA20ox*, in WT plants revealed the impact of GA on plant growth. This article suggests a new regulation model showing the regulative function of JA on GA.

Keywords

Calcium-dependent protein kinase; *Nicotiana attenuata*; jasmonic acid; gibberellic acid; plant morphology, plant development

Introduction

Hormone signaling plays critical roles in the physiology of multicellular organisms. In plants, auxin, gibberellic acids (GAs), jasmonic acid (JA), ethylene, brassinosteroids (BRs), abscisic acid (ABA), salicylic acid (SA), cytokinins (CKs), and strigolactones are all phytohormones that are essential for plant development, growth, and stress resistance. Among these, auxin, GAs, JA, ethylene, BRs, ABA, cytokinins, and strigolactones regulate plant development (Grant & Jones, 2009; Wolters & Jurgens, 2009; Lau & Deng, 2010; Depuydt & Hardtke, 2011; Krouk *et al.*, 2011), while JA, ethylene, SA, and ABA are well known for their functions in plant resistance to abiotic and biotic stresses (Maffei *et al.*, 2007; Browse, 2009; Grant & Jones, 2009; Wu & Baldwin, 2010). Given their strong impact on plant physiology, the biosynthesis, degradation, and the components of signaling networks of these phytohormones are tightly controlled, and mutants or genetically modified plants having altered levels of phytohormones or defects in hormone signaling generally exhibit abnormal growth, development and/or resistance to environmental stresses.

GAs play critical roles in seed germination, stem and leaf elongation, development of flowers, fruits, and seeds. Plants deficient in GAs cannot germinate without exogenously applied GAs and are dwarf and male sterile. The biosynthesis and signaling of GAs have been studied intensively. GAs are diterpene compounds synthesized from geranylgeranyl diphosphate through a complex pathway (Yamaguchi, 2008). Bioactive GAs bind to receptor GIBBERELLIN INSENSITIVE DWARF 1 (GID1) (Ueguchi-Tanaka *et al.*, 2005) and enhance the degradation of DELLA proteins through an F-box protein, SLEEPY1 (SLY1)-mediated ubiquitin-26S-proteasome pathway (Griffiths *et al.*, 2006; Ueguchi-Tanaka *et al.*, 2007). DELLA proteins are the master suppressors for GA-induced responses and thus control many aspects of development, growth, and even stress resistance (Sun TP *et al.*, 2010; Sun, XL *et al.*, 2011).

JA has been intensively studied for its important role in plant response to mechanical wounding and herbivore feeding (Wasternack, 2007; Wu & Baldwin, 2010). Using chloroplast membrane lipids, OPDA (12-oxo-phytodienoic acid) is formed in chloroplast, and OPDA is transported to peroxisome where it is finally converted to JA. At least eight enzymes are involved in this process (Wasternack, 2007). JA is further converted to JA-isoleucine conjugate (JA-Ile) in cytoplasm, which binds to an F-box protein, the receptor of JA-Ile, COI1 (CORONATINE INSENSITIVE 1) (Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2009). This leads to degradation of JAZ proteins, which are key transcription repressors of JA-dependent responses (Boter *et al.*, 2004; Chini *et al.*, 2007). JA is also required for normal

plant development. Not only plants having defects in JA production or signaling have dramatically compromised defense against herbivores and necrotrophic fungi (Vijayan *et al.*, 1998; Paschold *et al.*, 2007), but also exhibit male sterility, although they are generally normal during vegetative growth. In contrast, plants producing excessive amount of JA are stunted in growth and have decreased fertility (Bonaventure *et al.*, 2007; Hyun *et al.*, 2008) but show increased resistance to insect herbivores (Yang *et al*., in review).

A large body of evidence has indicated that none of these hormones functions without having cross-talks with others (Grant & Jones, 2009; Depuydt & Hardtke, 2011). For example, SA inhibits JA signaling through an NPR1-mediated pathway (Spoel *et al.*, 2003) and the gaseous hormone, ethylene, is also involved in the suppressing effect of SA on JA signaling (Leon-Reyes *et al.*, 2009). Auxin has cross-talks with cytokinins, ethylene, and BRs to regulate plant development (Swarup *et al.*, 2002). The cross-talks between GAs and other hormones have also been demonstrated. It is well known that GAs promote seed germination, while ABA antagonizes GAs by inhibiting germination (Finkelstein *et al.*, 2008). GAs and ABA signaling pathway may converge on DELLAs to modulate root development positive and negatively (Achard *et al.*, 2006). Furthermore, auxin interacts with gibberellin positively by promoting degradation of DELLAs or biosynthesis of GAs: Arabidopsis root elongation is stimulated by GAs; however, this process requires auxin, given that auxin promotes GA-induced degradation of DELLA proteins (Fu & Harberd, 2003).

JA biosynthesis and signaling have been studied in a wild tobacco plant, *Nicotiana attenuata*, for their functions in plant resistance to insect herbivores (Halitschke & Baldwin, 2003; Paschold *et al.*, 2007; Wang *et al.*, 2008). In *N. attenuata*, several genes were found to have a strong impact on JA accumulation, such as two mitogen-activated protein kinases (MAPKs), *SALICYLIC ACID-INDUCED PROTEIN KINASE* (*SIPK*) and *WOUND-INDUCED PROTEIN KINASE* (*WIPK*) (Wu *et al.*, 2007), *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*) (Yang *et al.*, 2011), *S-NITROSOGLUTATHIONE REDUCTASE* (*GSNOR*) (Wünsche *et al.*, 2011a), and *SUPPRESSOR OF G-TWO ALLELE OF SKP1* (*SGT1*) (Meldau *et al.*, 2011). In addition, *N. attenuata* calcium-dependent protein kinases (CDPKs), CDPK4 and CDPK5, play a redundant role in suppressing JA biosynthesis (Yang *et al.*, in review). *N. attenuata* silenced in both *CDPK4* and *CDPK5* (created using an inverted-repeat vector; hereafter, irCDPK4/5 plants) exhibit dramatically increased JA levels after wounding or insect feeding and compared with wild-type (WT), they show stunted stem elongation and abortion of flower buds, flowers, and young seed capsules (Yang *et al.*, in review).

Here we show that compared with the stems of WT, irCDPK4/5 plants contained highly increased levels of JA in stems, and genetic analysis indicated that the high JA contents in irCDPK4/5 accounted for the stunted stem elongation in irCDPK4/5. Exogenously applying bioactive GA_3 to irCDPK4/5 could largely rescue its defects in stem elongation. We further demonstrate that JA signaling suppressed the transcript levels of several GA biosynthetic genes and among which, *GA20ox* levels were drastically decreased. In contrast, *GA2ox*, which is important for deactivation of GAs, had elevated levels. We propose that JA signaling antagonizes the biosynthesis of GAs by repressing the transcription of GA biosynthetic genes and promoting GA deactivation genes.

Results

irCDPK4/5 plants have highly stunted stem elongation

Previously it has been shown that *N. attenuata* silenced in *CDPK4* and *CDPK5* has darker green leaves, highly decreased fertility, stunted stem growth; given that these phenotypes were observed in almost all independently transformed lines (Yang *et al.*, in review), in this study we focused on a single line irCDPK4/5-1 (hereafter irCDPK4/5). We germinated WT and irCDPK4/5 at the same time and grew them concurrently under standard glasshouse conditions and their growth was measured over time. Twenty-nine days after germination, when the stems of WT and irCDPK4/5 just started elongation, no large differences were found between the lengths of WT and irCDPK4/5 stems (both were ~ 2.5 cm); however, by 32 days after germination, irCDPK4/5 plants showed strong differences in stem elongation: irCDPK4/5 had more than 50% decreased stem lengths (Figure 1A). The diameters of irCDPK4/5 rosette leaves were similar to those of WT until 26 days after germination and thereafter irCDPK4/5 rosette leaves started to show delayed elongation (up to 15%) (Figure 1B). Thirty-five days after germination, WT rosette leaves stopped growth and those of irCDPK4/5 gradually reached almost the same sizes of WT rosette leaves (Figure 1B). Consistent with their reduced stem length, the average size of the pith cells of irCDPK4/5 plants was about 40% reduced in the longitudinal sections and 35% smaller in the cross sections, compared with those of WT stem pith (Figure 2).

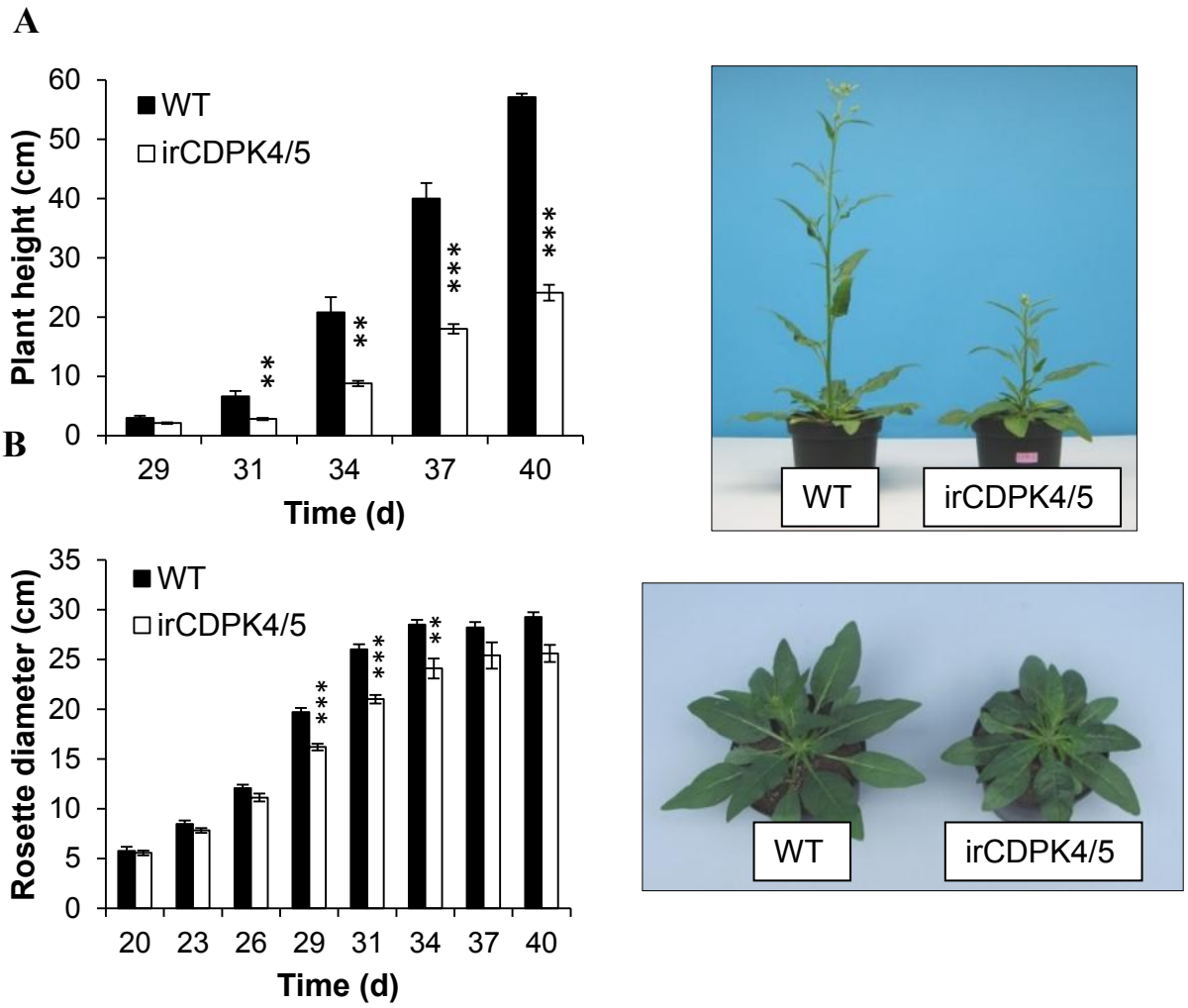


Figure 1. Silencing *CDPK4/5* results in stunted growth in *N. attenuata*.

Wild type (WT) and irCDPK4/5 plants were cultivated concurrently. Their heights (A) and diameters (B) (mean ± SE) were recorded until 40 days after germination Photos in (A) and (B) depicts 40 and 34days old plants. Asterisks indicate significant differences between WT and irCDPK4/5 plants (*t*-test; **, $P < 0.01$; ***, $P < 0.001$; $N = 5$).

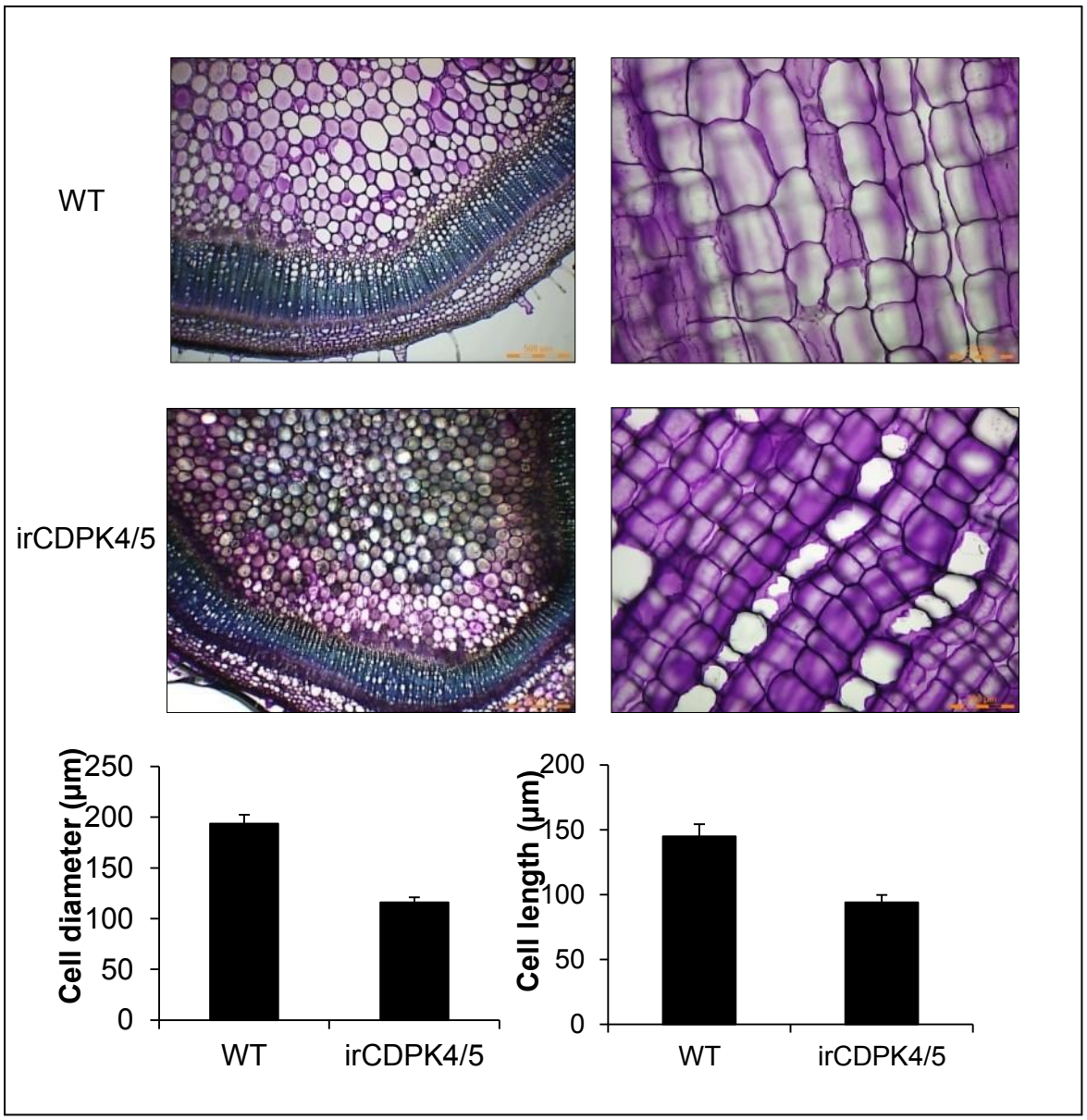


Figure 2. irCDPK4/5 plants have decreased cells sizes in stems.

Wild-type (WT) and irCDPK4/5 plants were cultivated concurrently. Stem sections were obtained from 40 days old plants and were stained with toluidine blue. Left panel: microscopy of stem cross sections and cell diameter (mean ± SE). Right panel: microscopy of stem longitudinal sections and cell length (mean ± SE).

Previously both *CDPK4* and *CDPK5* were found to be strongly expressed in *N. attenuata* stem (Yang *et al.*, in review). Analyzing the cross and longitudinal sections of plants expressing *CDPK4Pro:GUS* and *CDPK5Pro:GUS* (promoters of *CDPK4* and *CDPK5* fused with *β-glucuronidase*) indicated that in stems *CDPK4* was expressed in xylem, phloem, and cambium cells and *CDPK5* was expressed in xylem and cambium cells (Figure 3), supporting that CDPK4 and CDPK5 function in the stem of *N. attenuata*. Furthermore,

quantitative real time-PCR (qRT-PCR) indicated that the transcript levels of *CDPK4* and *CDPK5* did not show large changes during stem elongation (Figure S1), implying that the protein levels of CDPK4 and CDPK5 might be stable during the development of *N. attenuata* stem.

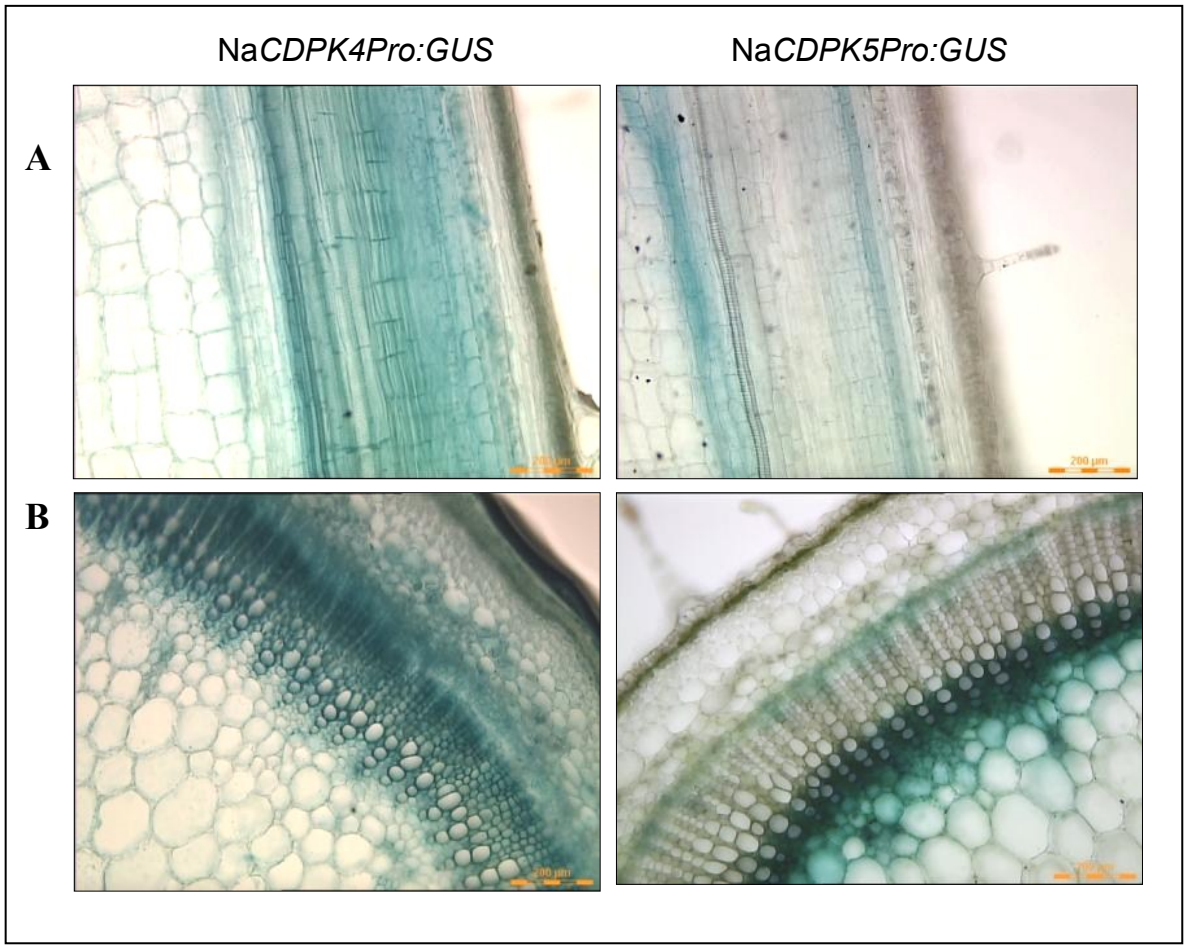


Figure 3. Expression of CDPK4 and of CDPK5 in stem.

Longitudinal (A) and cross (B) sections were obtained from stems of CDPK4Pro:GUS and CDPK5Pro:GUS plants. GUS staining is in blue (40 times magnification).

Silencing *CDPK4* and *CDPK5* results in dramatically increased contents of JA and secondary metabolites in stem

After wounding or herbivore attack, irCDPK4/5 produces very high levels of JA and even when untreated, the rosette leaves of irCDPK4/5 have 2-3 times greater JA contents than the leaves of WT (Yang *et al.*, in review). It was speculated that similar to the leaves of irCDPK4/5 plants the stems of irCDPK4/5 may accumulate high contents of JA. Thus, the levels of JA were quantified using a HPLC-MS/MS method. The JA concentrations in WT stems ranged from 2 to 12 ng g fresh mass (FM) $^{-1}$. Dramatically greater levels of JA were found in irCDPK4/5 stem: by day 41 and 48 after germination, stems of irCDPK4/5 plants had 240 and 140 fold greater JA than did stems of WT, respectively (Figure 4A). Greater JA levels were also found in 55- and 61-days-old plants (in senescence) (Figure 4A). Similarly, compared with those in WT leaves, increased JA levels were also found in the leaves of irCDPK4/5 in 34-, 41-, and 48-day-old plants, especially 41-day-old plants showed the strongest differences (irCDPK4/5 leaves contained 6 ng g FM^{-1}, while WT had 570 ng g FM^{-1}); however, no differences were found when plants were older than 48 days (Figure S2).

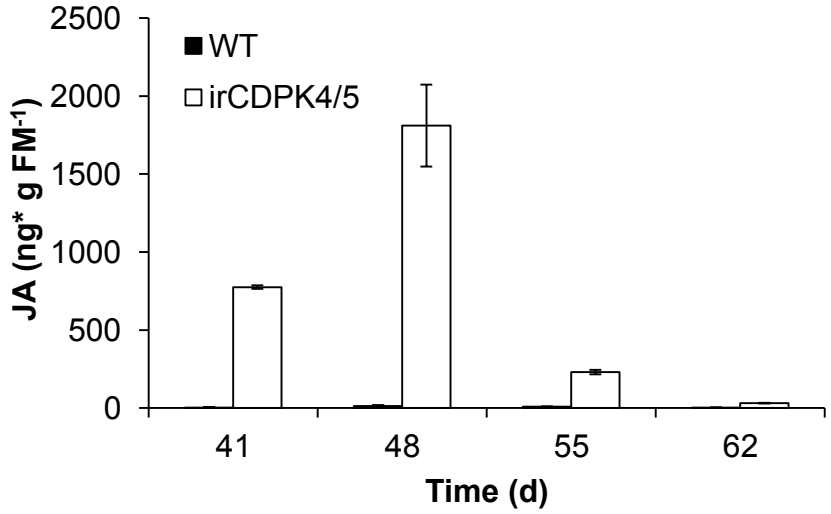
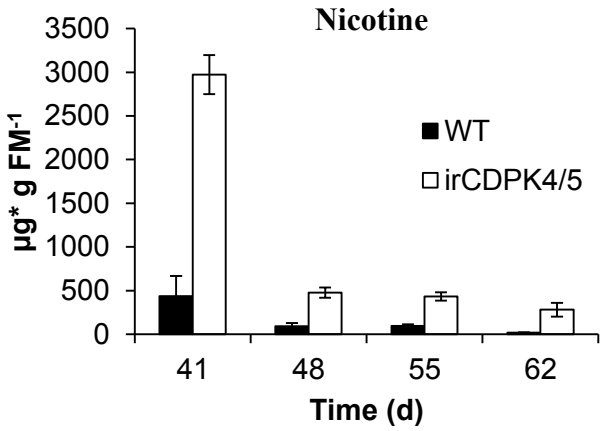


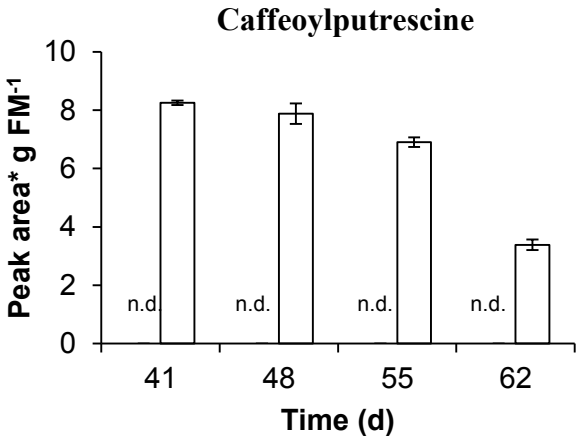
Figure 4. Contents of jasmonic acid in the stems of wild-type and irCDPK4/5 plants.

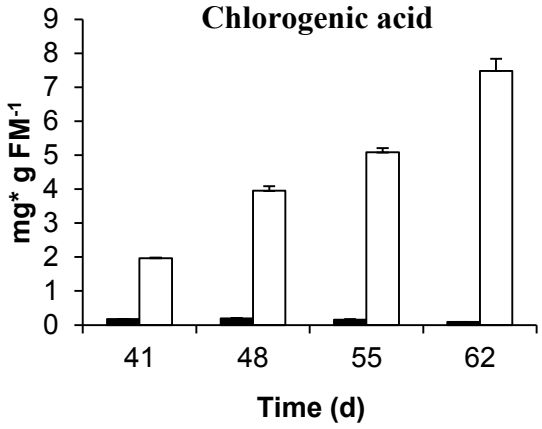
Wild-type (WT) and irCDPK4/5 plants were cultivated concurrently. The contents of JA (mean ± SE) were measured in the stems over 62 days after germination when plants almost completely reproduction (N = 5).

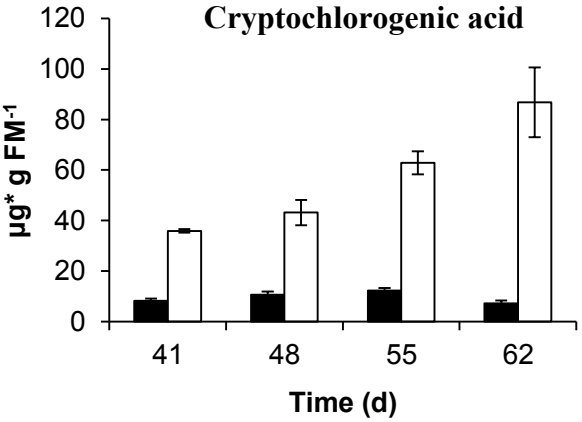
Enhanced JA production is usually associated with increased accumulation of plant secondary metabolites. Consistent with this scenario, we noticed that the stems of irCDPK4/5 plants rapidly turned brown when being exposed to air, whereas WT stems showed no

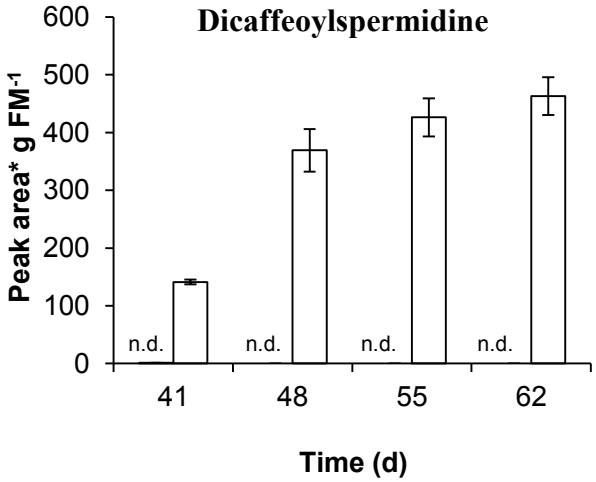
observable oxidation (Figure S3A), indicating that irCDPK4/5 may have elevated contents of phenolic compounds. Indeed, total phenolic contents increased 5 fold in irCDPK4/5 stems than in those of WT (Figure S3B). HPLC analysis indicated that WT stems had very little number of compounds (3 detectable peaks) whose concentrations were relatively low; in contrast, samples from the stems of irCDPK4/5 showed at least 11 peaks with relatively high signals (Figure S4). Among these peaks, six were found to be nicotine, caffeoylputrescine, chlorogenic acid, cryptochlorogenic acid, rutin, and dicaffeoylspermidine using standards, and another was identified to be N1-caffeoyl-N3-dihydrocaffeoylspermidine after fractionation and NMR analysis. In line with the highly increased JA levels in irCDPK4/5 stems, we found that by day 41 after germination, average nicotine content in WT was 436 μg g FM^{-1} and irCDPK4/5 had 2972 μg g FM^{-1}; although nicotine concentrations declined over time in both plants, 4-5 fold greater nicotine levels were still found in irCDPK4/5 (Figure 5A). Caffeoylputrescine was not detected in WT stems, but was found in all replicates of irCDPK4/5 (Figure 5B). Chlorogenic acid exhibited increasingly greater contents and by day 62, irCDPK4/5 had ~ 86 fold greater chlorogenic acid (7000 and 80 μg g FM^{-1} in irCDPK4/5 and WT, respectively) (Figure 5C). For cryptochlorogenic acid we measured a maximum of 85 μg g FM^{-1} in irCDPK4/5 at day 62 after germination, which was 12 fold higher compared to WT stem (Figure 5D). While dicaffeoylspermidine and N1-caffeoyl-N3-dihydrocaffeoylspermidine were hardly detected in WT stems, large peak areas of these compounds were found in the chromatograms from irCDPK4/5 stem samples (Figure 5, E and F). Notably, the concentrations of rutin were not so highly different between WT and irCDPK4/5 stems, although by day 62, rutin contents in irCDPK4/5 stems were still 5 fold greater (Figure 5G). Notably, compared with these compounds in leaves the irCDPK4/5, irCDPK4/5 stems contained greater amount of phenylpropanoid-polyamine conjugates, dicaffeoylspermidine and N1-caffeoyl-N3-dihydrocaffeoylspermidine (Figure S5).

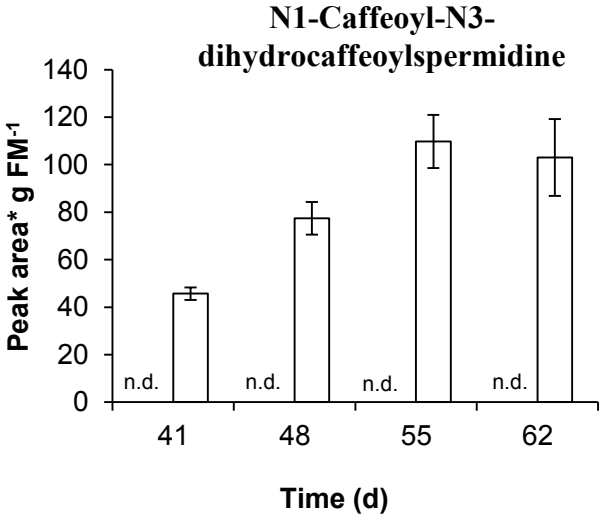

Nicotine
µg* g FM-1
3500
3000
2500
2000
1500
1000
500
0
WT
irCDPK4/5
41
48
55
62
Time (d)


Caffeoylputrescine
Peak area* g FM-1
10
8
6
4
2
0
n.d
n.d.
n.d.
n.d.
41
48
55
62
Time (d)


Chlorogenic acid
mg* g FM-1
9
8
7
6
5
4
3
2
1
0
41
48
55
62
Time (d)


Cryptochlorogenic acid
µg* g FM-1
120
100
80
60
40
20
0
41
48
55
62
Time (d)


Dicaffeoylspermidine
Peak area* g FM-1
600
500
400
300
200
100
0
n.d.
n.d.
n.d.
n.d.
41
48
55
62
Time (d)


N1-Caffeoyl-N3-dihydrocaffeoylspermidine
Peak area* g FM-1
140
120
100
80
60
40
20
0
n.d.
n.d.
n.d.
n.d.
41
48
55
62
Time (d)

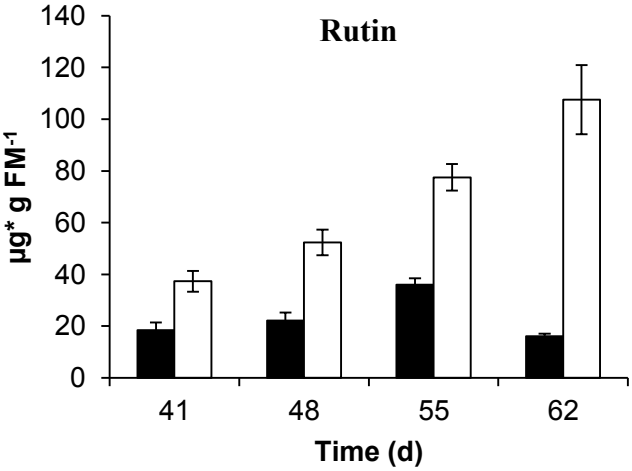

Rutin
µg* g FM-1
140
120
100
80
60
40
20
0
41
48
55
62
Time (d)

Figure 5. Secondary metabolite contents in the stems of wild-type and irCDPK4/5 plants.

Wild-type (WT) and irCDPK4/5 plants were cultivated concurrently. The contents (mean ± SE) of nicotine (A), caffeoylputrescine (B), chlorogenic acid (C), cryptochlorogenic acid (D), dicaffeoylspermidine (E), N1-caffeoyl-N3-dihydrocaffeoylspermidine (F), and rutin (G) were measured in the stems until 62 days after germination when plants almost completely reached reproduction stage (N = 5).

Highly elevated JA levels in irCDPK4/5 account for the inhibited stem elongation and accumulation of secondary metabolites

In Arabidopsis, several mutants that over-accumulate JA show stunted growth (Turner *et al.*, 2002; Hyun *et al.*, 2008; Bonaventure *et al.*, 2011). Thus, we hypothesized that the stunted growth resulted from the high JA levels in irCDPK4/5 plants.

To examine this hypothesis, first we applied methyl jasmonate (MeJA) to the stems of WT *N. attenuata* and recorded the stem lengths over time. As expected, applying MeJA to stems did inhibit the elongation of stems (Figure 6A). A genetic approach was also employed: irCDPK4/5 was crossed with the irAOC line, which is silenced in the allene oxide cyclase (AOC), an important gene encoding a JA biosynthetic enzyme (Kallenbach *et al.*, in review). Analyzing the JA contents in these crossed plants (irCDPK4/5×irAOC) indicated that silencing *AOC* effectively reduced their JA levels of irCDPK4/5 to those of WT or irAOC (from 1500 ng g FM^{-1} to 50 ng g FM^{-1}) (Figure 6B). Importantly, the development of leaves and stems in irCDPK4/5×irAOC plants was completely restored to that of WT or irAOC plants (Figure 6B inset). The same results were also found when irCDPK4/5 was crossed with an irCOI1 line (Paschold *et al.*, 2007), whose *COI1* (the receptor in JA signaling) was silenced, or with an ovJMT line, which ectopically expressed an Arabidopsis *JMT* gene (*JASMONIC ACID O-METHYLTRANSFERASE*). In ovJMT plants, JMT rapidly converts JA to MeJA, which is dissipated to the headspace, and functions as a metabolic sink of JA; thus ovJMT plants show JA-deficient phenotypes (Stitz *et al.*, 2011).

In addition, when JA biosynthesis is suppressed by silencing *AOC*, the highly increased contents of secondary metabolites in irCDPK4/5 stems also declined to those in WT or irAOC plants (Figure 6C). Similarly, silencing *COI1* or overexpression of JMT also abolished the accumulation of these compounds in irCDPK4/5 stem (Figure S6).

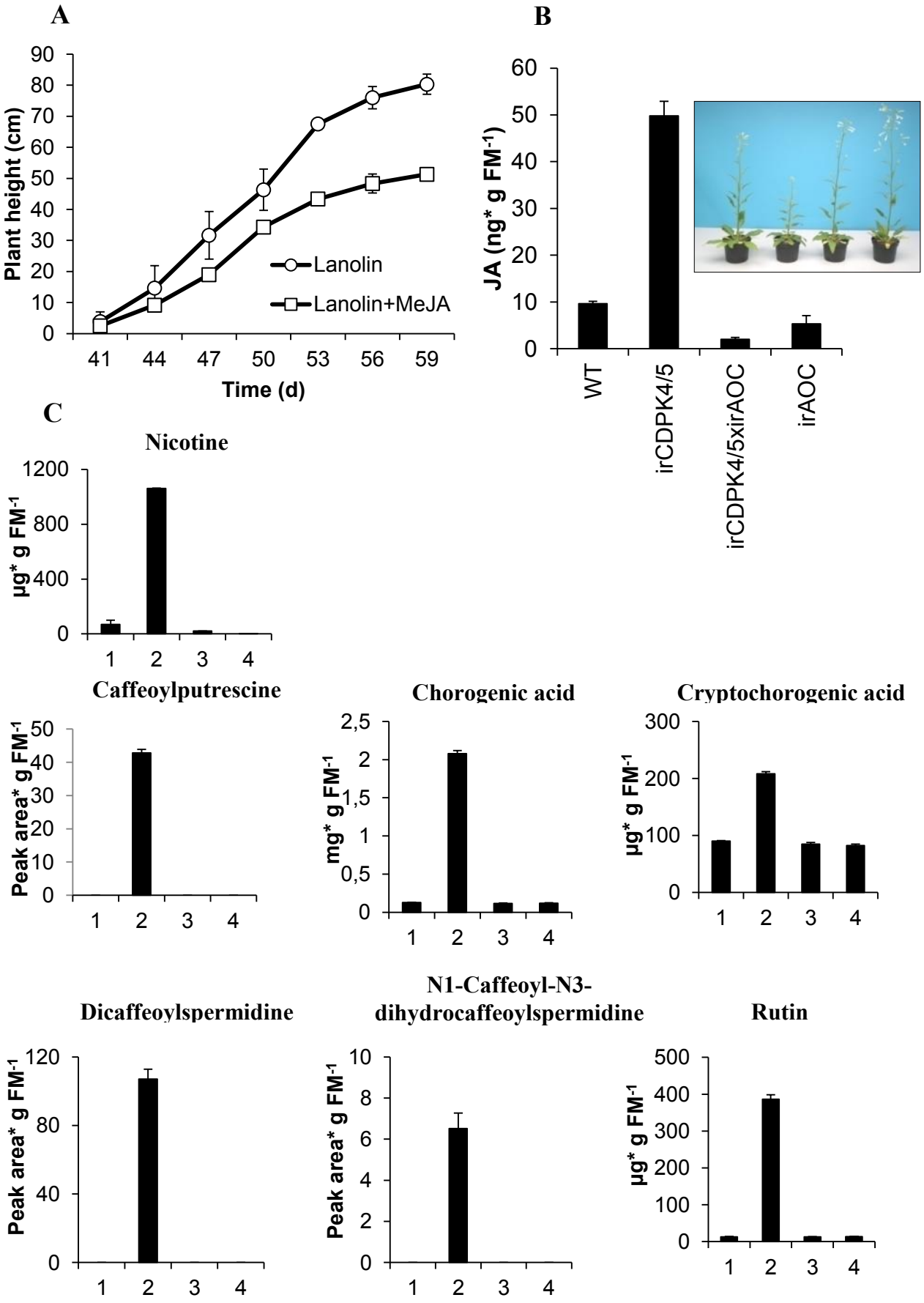

A
Plant height (cm)
Time (d)
Lanolin
Lanolin+MeJA
B
JA (ng* g FM-1)
WT
irCDPK4/5
irCDPK4/5xirAOC
irAOC
C
Nicotine
µg* g FM-1
Caffeoylputrescine
Peak area* g FM-1
Chorogenic acid
mg* g FM-1
Cryptochorogenic acid
µg* g FM-1
Dicaffeoylspermidine
Peak area* g FM-1
N1-Caffeoyl-N3-dihydrocaffeoylspermidine
Peak area* g FM-1
Rutin
µg* g FM-1

Figure 6. Applying MeJA to *N. attenuata* suppresses growth and abolishing JA biosynthesis in irCDPK4/5 restores stem elongation and decreased the contents of secondary metabolites.

(A) *N. attenuata* stems were treated with MeJA (100 μg in 20 μL of lanolin) every 3 days after bolting. Plants supplied with pure lanolin pastes (20 μL) served as comparisons. The plant heights (mean ± SE) were measured (N = 5). (B) JA concentration (mean ± SE) in the stems of 41 days old wild-type (WT), irCDPK4/5, irCDPK4/5×irAOC, and irAOC plants (N = 5). Inset: a photograph of 47 days old WT, irCDPK4/5, irCDPK4/5×irAOC, and irAOC plants (left to right). (C) Contents (mean ± SE) of secondary metabolites in WT (1), irCDPK4/5 (2), irCDPK4/5×irAOC (3) and irAOC (4) stem tissues (N = 5).

Consistent with our data that over-accumulated JA inhibits stem elongation, irAOC, irCOI1, and ovJMT plants which had compromised JA biosynthesis, signaling, and accumulation, respectively, exhibited about 20% longer stems than did WT, 38 days after germination (Figure S7).

Thus, we concluded that the drastically elevated contents of JA in irCDPK4/5 were the reason for the stunted growth of stems and the high contents of secondary metabolites.

Mis-allocation of carbon source does not likely account for the inhibited stem growth in irCDPK4/5 plants

It is possible that the highly enhanced accumulation of secondary metabolism diverted the limited carbon sources from being converted to growth-related compounds and thus resulted in stunted elongation of stems in irCDPK4/5.

To examine this possibility, irCDPK4/5 plants were crossed with the irMYB8 line to form irCDPK4/5×irMYB8. Transcription factor MYB8 plays a critical role in regulating the biosynthesis of certain major phenolic compounds and phenylpropanoid-polyamine conjugates, such as chlorogenic acid, caffeoylputrescine, and dicaffeoylspermidine (Kaur *et al.*, 2010; Onkokesung *et al*., 2012). HPLC analysis confirmed that crossing irCDPK4/5 with irMYB8 effectively abolished the accumulation of caffeoylputrescine and chlorogenic acid (Figure 7) and decreased the contents of rutin, dicaffeoylspermidine, N1-Caffeoyl-N3-dihydrocaffeoylspermidine, and nicotine 5, 3.5, 5, and 6.5 fold, respectively (Figure 7) Cryptochlorogenic acid levels did not differ between irCDPK4/5 and irCDPK4/5xirMYB8 stem tissue (Figure 7). However, compared with WT or irMYB8, irCDPK4/5×irMYB8 were even 60% smaller then irCDPK4/5 plants, indicating that decreasing the accumulation of these compounds had no effect on restoring the growth of irCDPK4/5 stems.

Thus, it is unlikely that carbon mis-allocation resulted in the stunted stem growth in irCDPK4/5.

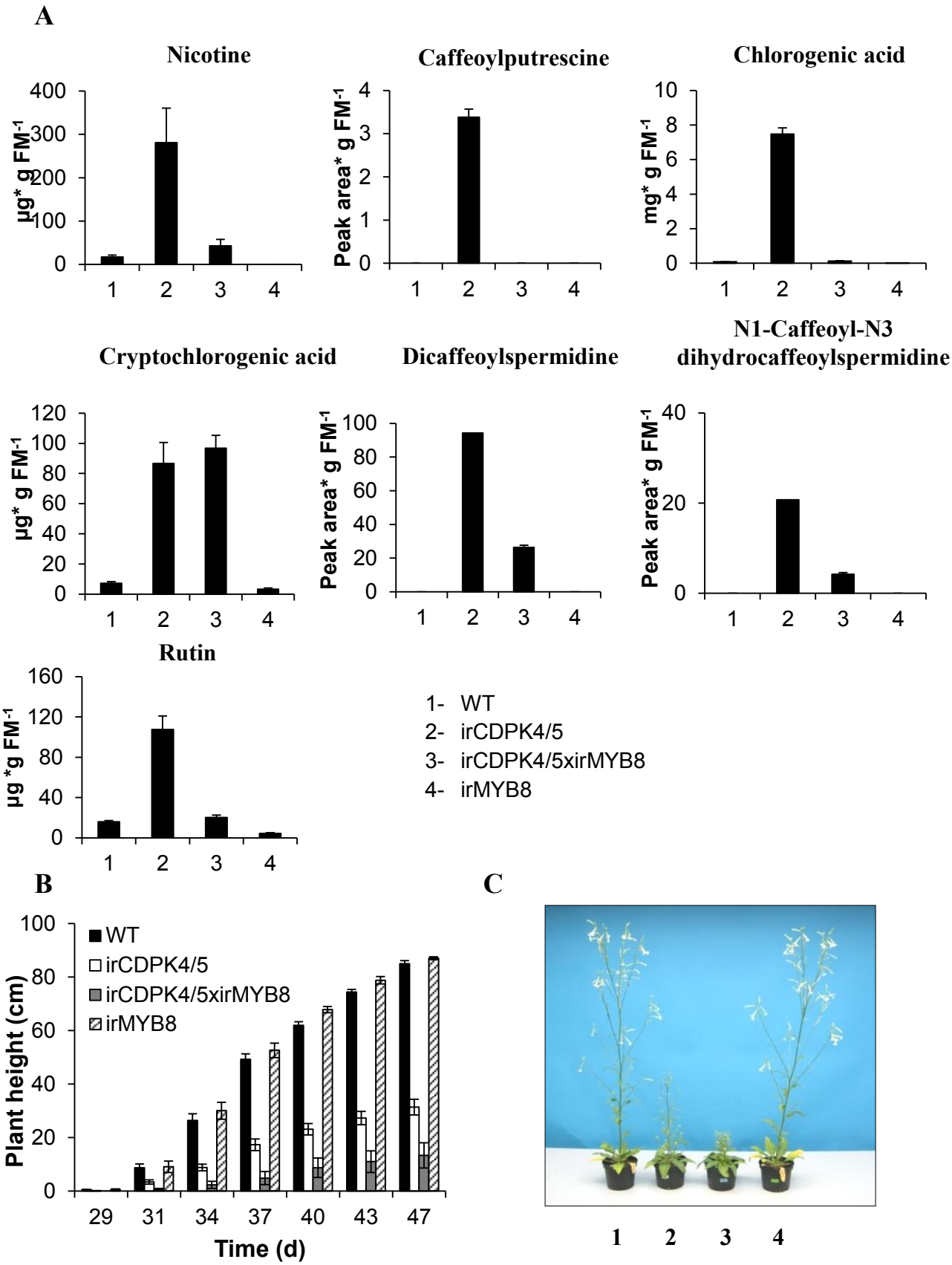

A
Nicotine
µg* g FM-1
Caffeoylputrescine
Peak area* g FM-1
Chlorogenic acid
mg* g FM-1
Cryptochlorogenic acid
µg* g FM-1
Dicaffeoylspermidine
Peak area* g FM-1
N1-Caffeoyl-N3 dihydrocaffeoylspermidine
Peak area* g FM-1
Rutin
µg *g FM-1
1- WT
2- irCDPK4/5
3- irCDPK4/5xirMYB8
4- irMYB8
B
Plant height (cm)
WT
irCDPK4/5
irCDPK4/5xirMYB8
irMYB8
Time (d)
C
1 2 3 4

Figure 7. Silencing *MYB8* in irCDPk4/5 decreases contents of most of the detected secondary metabolites.

(A) The contents (mean ± SE) of nicotine, caffeoylputrescine, chlorogenic acid, cryptochlorogenic acid, dicaffeoylspermidine, N1-caffeoyl-N3-dihydrocaffeoylspermidine, and rutin in wild-type (WT), irCDPK4/5, irCDPK4/5×irMYB8, and irMYB8. (B) Plant heights (mean ± SE) of WT, irCDPK4/5, irCDPK4/5×irMYB8, and irMYB8 over 47 days after germination. (C) A photograph of 50-day-old plants.

Complementation of GA to irCDPK4/5 plants recovers stem growth

The stunted growth of irCDPK4/5 stems and their decreased cell sizes are largely reminiscent of the phenotypes of gibberellin deficient or insensitive plants (Sun & Gubler, 2004). Thus, bioactive GA_3 was applied to irCDPK4/5 to determine if exogenous supplying GA could recover the growth of irCDPK4/5 stems. Twenty-days after germination, when plants were still at early rosette stage, both WT and irCDPK4/5 plants were supplied daily with 3 μM GA_3 for 28 days; plants treated with the solvent (water containing 0.01% ethanol) were used for comparisons. WT plants did not grow taller after GA_3 treatment, except that WT plants showed larger stem leaves (Figure 8). Importantly, GA_3 supplementation largely restored the growth of irCDPK4/5 plants, reaching 80% of the height of WT (Figure 8). These data suggest that deficiency of GAs likely resulted in the stunted growth of irCDPK4/5 stem

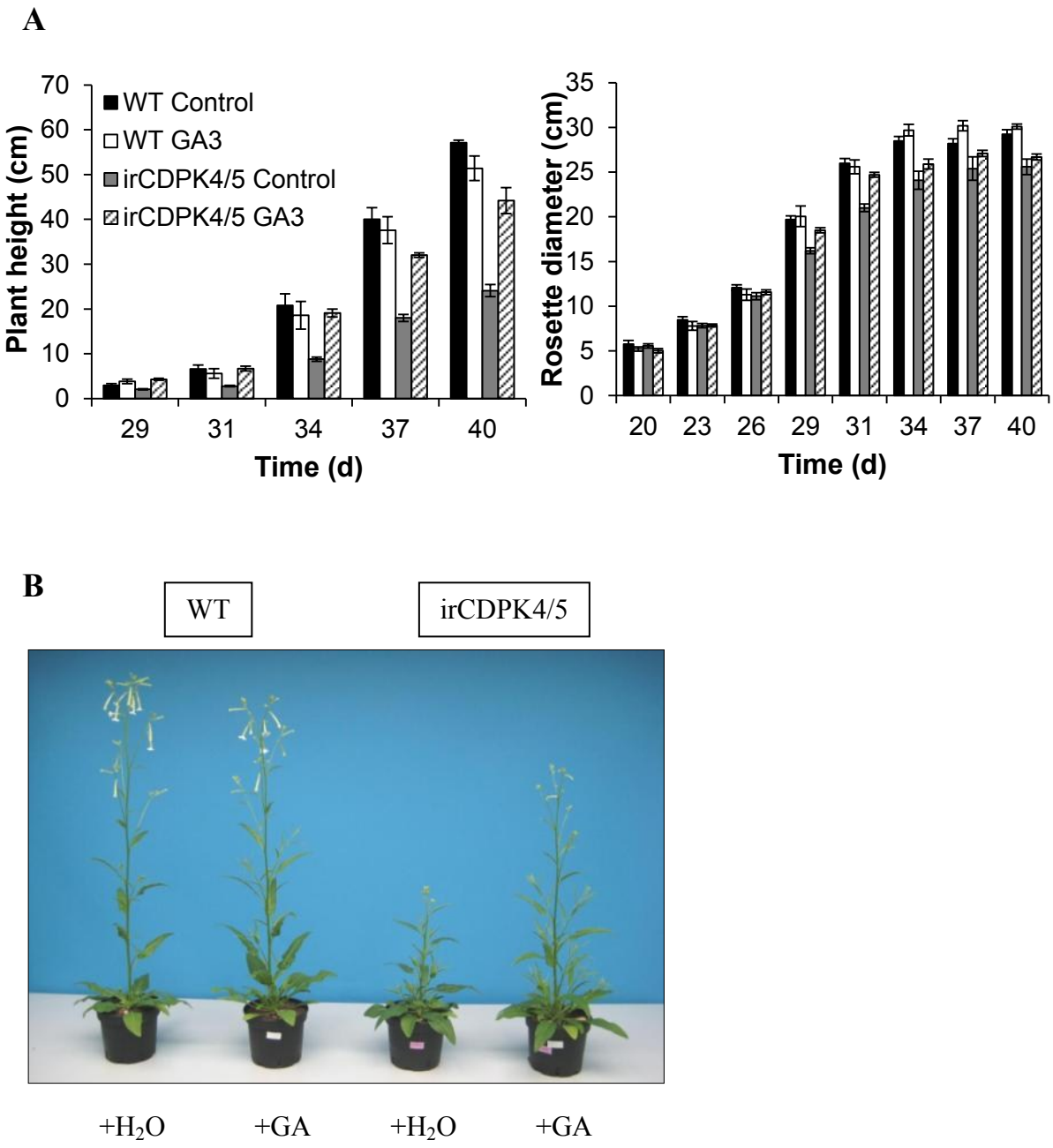


Figure 8. Exogenous application of GA_3 largely rescues the growth of irCDPK4/5 stems.

Wild-type (WT) and irCDPK4/5 were grown concurrently. GA_3 (3 μM) was sprayed to plants once a day, and plants sprayed with water containing 0.01% ethanol were used for comparisons. (A) Plant heights and rosette diameters (mean ± SE) of WT and irCDPK4/5 plants over 40 days after germination. (B) A photograph of WT and irCDPK4/5 plants 41 days after germination, which were treated with either water or GA_3.

Highly elevated JA levels suppress the transcript levels of GA biosynthetic genes, especially *GA20ox*, but promote the transcripts of GA deactivating gene *GA2ox*

The biosynthesis of GAs has been intensively studied (Yamaguchi, 2008). It starts from GERANYLGERANYL DIPHOSPHATE (GGDP), a common C_{20} precursor for diterpenoids; *ent*-COPALYL DIPHOSPHATE SYNTHASE (CPS) and *ent*-KAURENE SYNTHASE (KS) convert GGDP to *ent*-kaurene. Sequentially, *ent*-KAURENE OXIDASE (KO) and *ent*-KAURENOIC ACID OXIDASE (KAO) catalyze the formation of GA_{12} from *ent*-kaurene. GA_{12} is further converted to various forms of active GAs (GA_1, GA_3, and GA_4) by GA 20-OXIDASE (GA20ox), GA 3-OXIDASE (GA3ox), and the unidentified GA 13-OXIDASE (GA13ox). Moreover, GA 2-OXIDASES (GA2oxs) are involved in deactivating GAs. To gain further insight into the mechanism by which the high concentration of JA in irCDPK4/5 antagonized the levels of GAs, we identified the close homologues of these genes in a transcriptome database obtained from 454 sequencing of RNAs isolated from pooled various tissues of *N. attenuata*, including root, stem, and leaf (Figure S8). Subsequently, qRT-PCR was performed to determine their transcript levels.

In stems of WT and irCDPK4/5 plants, several genes involved in GA biosynthesis, deactivation, and signaling showed substantial differences in transcript levels (Figure 9). Compared with those in WT stems, in the stems of irCDPK4/5 plants, genes encoding enzymes in the early steps of GA biosynthesis, *CPS*, *KS*, *KO*, and *KAO*, showed no changes (for *CPS*), only ~ 20 and 30% reduction (for *KS* and *KO*, respectively), or even more than 2 fold greater (for *KAO* gene) in transcript levels (Figure 9A to 9D). Strikingly, the transcript levels of *GA20ox* gene in irCDPK4/5 stems were only 2% of those in WT stems (Figure 9E). GA20ox and GA3ox catalyze the last steps of GA biosynthesis. *GA3ox* transcript levels were 1.7 fold increased in irCDPK4/5 stems (Figure 9E). Unexpectedly, we found that the transcript abundance of *GA2ox*, a gene involved in GA catabolism, was 2.7 fold greater in irCDPK4/5 stems than in WT stems (Figure 9G). Rice *gid1* mutant, which has defect in receptor gene GID1 (*GIBBERELLIN INSENSITIVE DWARF1*), has remarkably high levels of GAs (about 100 times more than WT rice) (Ueguchi-Tanaka *et al.*, 2005), and supplying GAs to Arabidopsis suppresses the transcript abundance of all three GIDs isoforms (Griffiths *et al.*, 2006), suggesting a feedback regulation between GA perception and biosynthesis. Two GA receptor genes (*GID1a/c* and *GID1b*) were found in *N. attenuata* transcriptome database. While *N. attenuata GID1a/c* had no difference in transcript levels between irCDPK4/5 and WT stems, *GID1b* transcript abundance in irCDPK4/5 stems was 11 times elevated (Figure 9H).

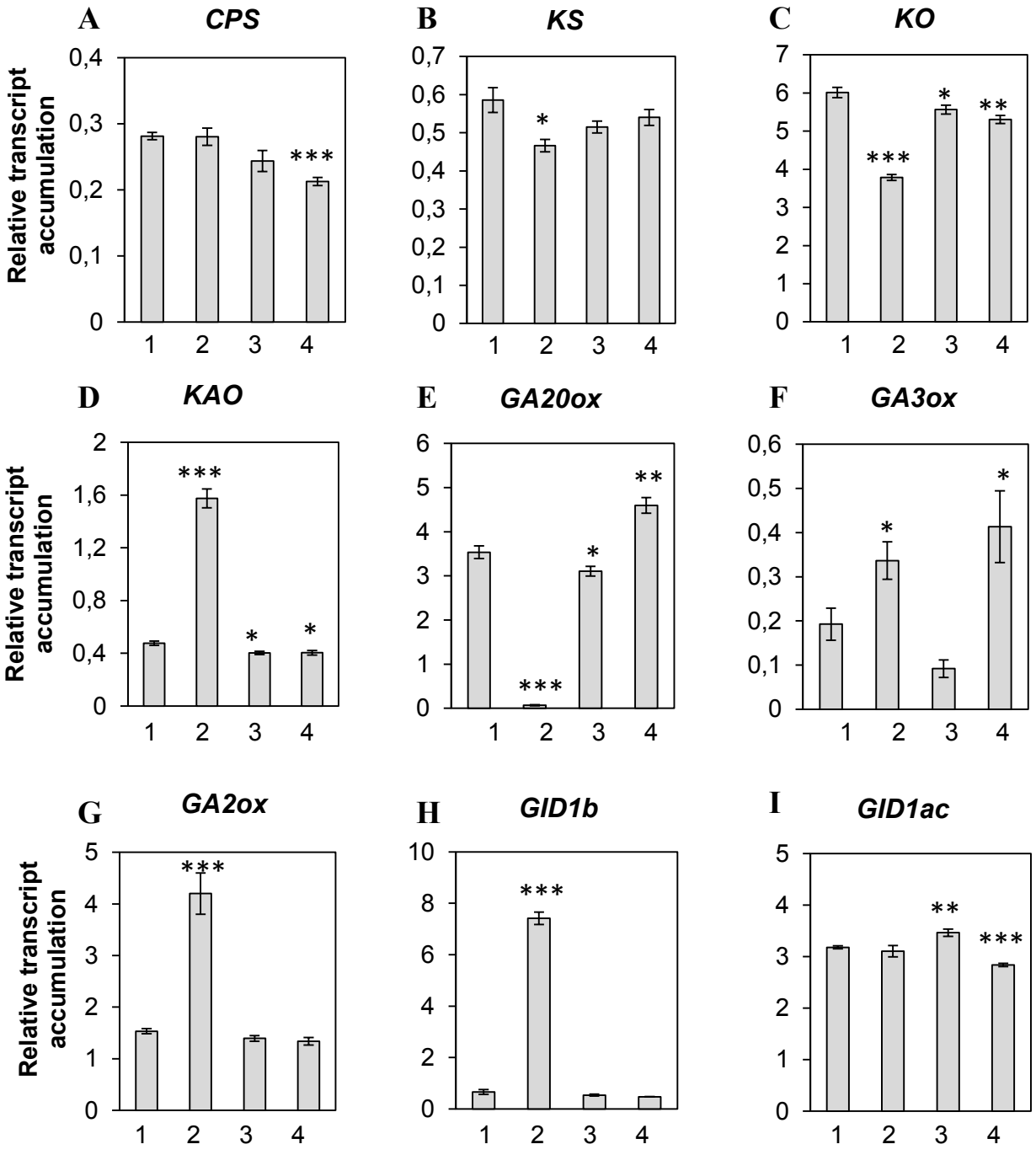

A
CPS
B
KS
C
KO
D
KAO
E
GA20ox
F
GA3ox
G
GA2ox
H
GID1b
I
GID1ac
Relative transcript accumulation
1- WT
2- irCDPK4/5
3- irCDPK4/5xirAOC
4- irAOC

Figure 9. Relative transcript accumulation of GA biosynthetic and signaling genes in wild-type, irCDPK4/5, irCDPK4/5×irAOC and irAOC stem tissues.

Wild-type (WT), irCDPK4/5, irCDPK4/5×irAOC and irAOC plants were grown concurrently. Stems from 41 days old plants were harvested and qRT-PCR was done to examine the transcript levels (mean ± SE) of (A) *CPS*, (B) *KS*, (C) *KO*, (D) *KAO*, (E) *GA20ox*, (F) *GA3ox*, (G) *GA2ox*, (H) *GID1B*, and (I) *GID1AC* gene. Asterisks indicate significant differences between WT and irCDPK4/5, irCDPK4/5×irAOC or irAOC plants (*t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; $N = 5$).

Importantly, compromising the accumulation of JA in irCDPK4/5 by crossing it with irAOC plants almost fully reversed the transcript levels of all these genes (Figure 9A to 9I). This confirmed that the highly accumulated JA in irCDPK4/5 plants accounted for the altered transcriptional levels of these GA-related genes. Furthermore, consistently with the profile of JA contents in stems over time, we found that the transcript levels *GA20ox* were most strongly suppressed in 48 days old irCDPK4/5 plants, when they had highest amount of JA in stems (Figure S9). Notably, decreasing the JA levels in irAOC plants also led to enhanced transcript abundance of *GA20ox* and *GA3ox* (17 and 114%, respectively, compared with those in WT stems) (Figure 9E and 9F).

Recently, Dayan *et al*. (2012) demonstrated that in tobacco (*Nicotiana tabacum*) leaves are required for the accumulation of bioactive GAs in the stem and consequently for normal stem elongation. Thus, we also determined the transcript levels of these GA biosynthetic genes in leaves. No large differences were found, except that *GA20ox*, *GA3ox*, and *GA2ox* showed 35, 60, and 47% decreased transcript levels in irCDPK4/5 than in WT leaves (Figure S10). However, compared with those in WT leaves, *GID1B* transcript levels were slightly decreased in irCDPK4/5, in contrast to the highly upregulated expression of *GID1B* in irCDPK4/5 stems.

GA20ox plays an important role in *N. attenuata* stem growth

Given the huge differences between the transcript levels of *GA20ox* in WT and irCDPK4/5 plants and its critical role in GA biosynthesis, it was inferred that JA antagonizes the biosynthesis of GAs mainly by inhibiting the transcript accumulation of *GA20ox* gene. To further confirm that decreased transcript levels of *GA20ox* affect stem elongation, a virus-induced gene silencing (VIGS) approach was used to knock down *GA20ox*: Partial sequence of *GA20ox* was cloned into vector pTV00 to form pTV-GA20ox, and *Agrobacterium* harboring pTV-GA20ox was subsequently inoculated into *N. attenuata* to silence *GA20ox*.; plants inoculated with *Agrobacterium* carrying pTV00 (empty vector) were used for comparisons (VIGS-GA20ox and EV plants, respectively). qRT-PCR analysis indicated that the transcript abundance of *GA20ox* in the stems of VIGS-GA20ox plants was around 18% of that in the stems of EV plants (Figure 10). As expected, compared with those of EV plants,

VIGS-GA20ox exhibited reduced stem lengths (~ 40%) (Figure 10A), confirming that GA20ox plays an important role in stem elongation. Silencing efficiency in GA20ox silenced plants was 18%, but still enough to show a significant stem growth reduction (Figure 10B).

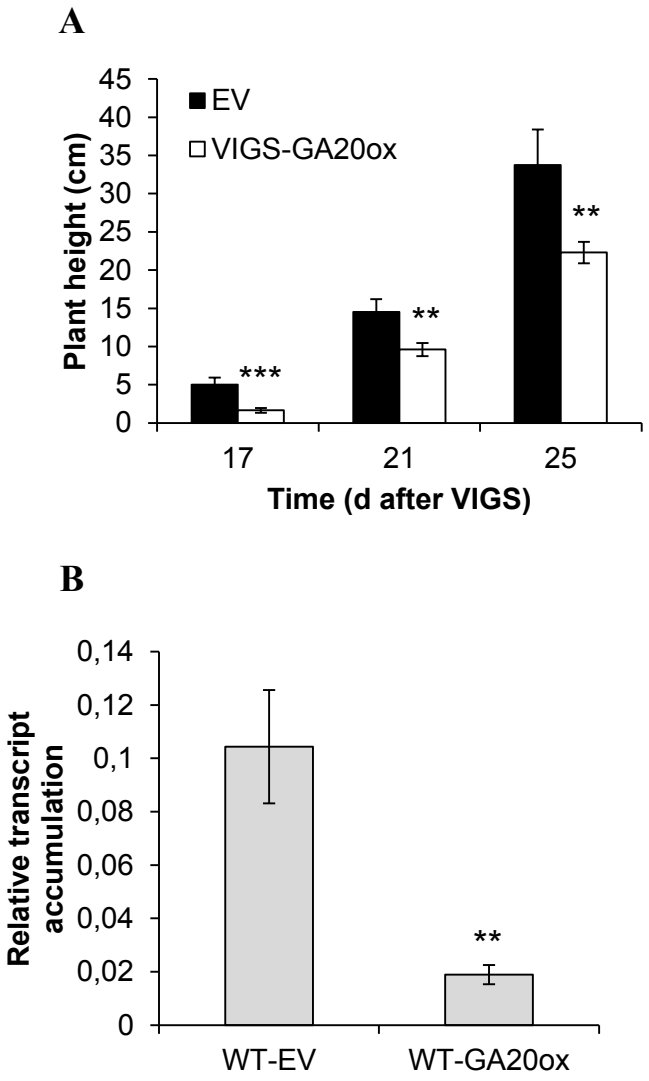


Figure 10. Silencing of *GA20ox* gene in *N. attenuata* results in stunted stem elongation.

GA20ox was silenced in *N. attenuata* using a virus-induced gene silencing system (VIGS-20ox plants). Plants inoculated with *Agrobacterium* carrying empty vector were used as comparisons (EV plants) (A) Plant heights (mean ± SE; N = 5) of EV and VIGS-GA20ox plants. (B) Transcript levels of *GA20ox* in EV and VIGS-GA20ox plants. Asterisks indicate significant differences between WT and irCDPK4/5 plants (*t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; N = 5).

Discussion

Plant development, growth, and stress responses are largely orchestrated by various hormone pathways, and these pathways often have synergistic or antagonistic cross-talks on certain aspects of plant physiology (Grant & Jones, 2009; Depuydt & Hardtke, 2011). Using irCDPK4/5 plants, which have lost the suppressing function of CDPK4 and CDPK5 and thus have dramatically increased levels of JA, we show that JA signaling repress the biosynthesis of GAs by inhibiting the transcription of several GA biosynthetic genes, especially *GA20ox*, a key enzyme catalyzing formation of bioactive GAs.

CDPK4 and CDPK5 are critical repressors of JA biosynthesis in stems and leaves

Although almost all enzymes in JA biosynthesis have been cloned (Wasternack, 2007), still little is known about how JA biosynthesis is regulated. In *N. attenuata*, although the underlying mechanisms remain unclear, MAPKs (Wu *et al.*, 2007), receptors BRI1 and COI1 (Paschold *et al.*, 2008; Yang *et al.*, 2011), nitric oxide signaling (Wünsche *et al.*, 2011a; Wünsche *et al.*, 2011b), and co-chaperon SGT1 (Meldau *et al.*, 2011) all influence JA levels. CDPKs are activated after binding of Ca^{2+}. In Arabidopsis, a mutant carrying mis-sense mutation in *TWO PORE CHANNEL 1* gene (*TPC1*), which encodes a Ca^{2+}-permanent non-selective cation channel, high amount of JA accumulates, implicating Ca^{2+} functions in JA biosynthesis (Bonaventure *et al.*, 2007). Thus, we speculate that in *N. attenuata* stems and leaves Ca^{2+} and CDPK4/5 are both important for maintaining normal levels of JA. In another solanaceous plant, tomato, *AOC* gene (encodes a key enzyme in JA biosynthesis) is expressed in vascular bundles (Stenzel *et al.*, 2003). Consistent with the function of CDPK4/5 in stems and in JA biosynthesis, we detected strong activity of CDPK4/5 promoters in the vascular cells in stems.

Compared with WT *N. attenuata*, simultaneously silencing *CDPK4*/*5* increases wounding- and *M. sexta* herbivory-induced JA about 5.25 and 2.4 fold respectively (Yang *et al*., in review). Here we also show that CDPK4/5 not only negatively affect these stress-induced JA biosynthesis but also are involved in suppressing accumulation of JA in *N. attenuata* leaves and stems (Figure 4 and Figure S2). However, compared with their roles in controlling wounding- and herbivory-induced JA, their functions in suppressing the biosynthesis of JA in stems and leaves seem likely to be more important, given that irCDPK4/5 exhibited a hundreds time greater JA contents than did WT. Furthermore, in stems the JA contents irCDPK4/5 were likely associated with plant age – JA contents were more dramatically greater during stem elongation (usually between 33 to 50 days after germination) than did older plants when stem growth was almost terminated. Similar dependency was also

observed for leaves, 41-day-old leaves of irCDPK4/5 exhibited very high JA levels, while compared with WT leaves, younger or older ones showed much smaller differences in JA contents. Moreover, CDPK4/5 seem to play an more important role in stems than in leaves, considering that the very high levels of JA appeared more transiently in leaves. It is likely that during the rapidly stem and leaf elongation process, CDPK4/5 are important suppressors that mediate JA homeostasis and therefore relieve the antagonistic effect of JA on GA biosynthesis to supply normal amount of GAs for cell expansion in these organs.

The importance of CDPK4/5 in maintaining JA homeostasis is also demonstrated by the over-accumulation of secondary metabolites in irCDPK4/5 stems. In WT plants, compared with leaves, stems contain very little amount of secondary metabolites, even after leaves are treated with wounding or herbivory stress (Hettenhausen, Heinrich, Baldwin, and Wu, unpublished data). However, several compounds, which are normally non-detectable in stems, highly accumulated in irCDPK4/5 stems, and this was due to overly produced JA, since silencing *AOC* in irCDPK4/5 completely reversed the contents of these compounds to those in WT or irAOC plants. Moreover, these data also suggest that under certain conditions, stems are able to synthesize secondary metabolites, which are usually produced in other organs, and compounds could even be produced in high quantity (Figure S5). The possibility that these compounds were produced in leaves and somehow transported to stems cannot be ruled out.

JA antagonizes biosynthesis of GAs

At least three Arabidopsis mutants *cev1*, *fou2*, and *dgl-D* produce excessive amount of JA, and these plants all exhibit stunted growth (Ellis *et al.*, 2002; Bonaventure *et al.*, 2007; Hyun *et al.*, 2008). Crossing *dgl-D* with *opr3* or *coi1* to abolish its JA biosynthesis or signaling effectively restored its normal vegetative growth (Hyun *et al.*, 2008). Similarly, silencing *ACO1* or *COI1* or ectopically overexpressing *JMT* gene in irCDPK4/5 also reversed its stem elongation. Thus, high levels of JA inhibit plant growth and importantly, the defect in the growth of irCDPK4/5 resulted only from over-accumulated JA but not from other pathways that CDPK4 and/or CDPK5 may regulate.

One possible scenario that JA inhibits plant growth is that JA may increase the allocation of carbon and nitrogen resources to defense/secondary metabolites thereby reduce growth. When irCDPK4/5 crossed with irMYB8 (deficient in an important transcription factor for biosynthesis of phenolic compounds and phenylpropanoid-polyamine conjugates), we could effectively decrease the contents of most of the detectable secondary metabolites, but irCDPK4/5×irMYB8 did not restore normal growth and was even smaller than irCDPK4/5.

Therefore, it is very likely that carbon mis-allocation only account little for the decreased stem elongation of irCDPK4/5.

Furthermore, the responsiveness of irCDPK4/5 to exogenously applied GA_3 ruled out that JA has an inhibitory effect on GA signaling. Conversely, probably due to the feedback regulatory pathway between GAs and GID1 (receptors for GA), it might be that the suggested decreased GA contents in irCDPK4/5 induced several fold increase of *GID1b* transcripts; therefore, irCDPK4/5 may have greater sensitivity to GAs than did WT plants, and this might be the reason why irCDPK4/5 responded strongly to the supplied GA_3, while WT plant did not. Importantly, transcriptional analysis indicated that over-accumulated JA specifically suppressed the transcript levels of *GA20ox*, a key gene in GA biosynthesis: qRT-PCR indicated that irCDPK4/5 stems had only 2% of the *GA20ox* transcripts in WT stems. Although changes of transcript levels in other genes may also have contributed to the GA contents in irCDPK4/5 plants, e.g., the decreased *KO* and the increased *GA2ox* gene (encoding a GA deactivating enzyme), only *GA20ox* showed such a huge difference between WT and irCDPK4/5 (Figure 9E). Using VIGS system, we further confirmed that *GA20ox* is important for promoting the growth of *N. attenuata*. Results from crossing irCDPK4/5 with irAOC or ovJMT (impaired in JA accumulation) or with irCOI1 (compromised in JA signaling) consistently indicated that high JA contents suppress GA biosynthesis by repressing the transcript levels of *GA20ox* through a COI1-mediated pathway. Under optimal growth conditions, plants deficient in JA or JA signaling are often taller than WT plants (Figure S8). Compared with WT, JA-deficient irAOC plants had more than 1 fold greater transcripts of *GA3ox* (Figure 9F), and this may lead to slightly increased bioactive GAs and thereby enhance plant growth.

It is unclear how JA signaling suppresses transcript levels of *GA20ox*. Besseau *et al.* (2007) found that in Arabidopsis over-accumulation of flavonoids leads to inhibited auxin transport and reduced plant size. Recently, it was found that in roots of Arabidopsis seedlings, MeJA inhibits endocytosis and accumulation of PIN2 (PIN-FORMED 2), which is an auxin efflux carrier, (Sun, JQ *et al.*, 2011). Furthermore, an important transcript factor regulated by JA, MYC2, binds to the promoters of *PLETHORA1* (*PLT1*) and *PLT2*, thereby suppresses their expression and results in decreased auxin-induced regulation of stem cell niche maintenance (Chen *et al.*, 2011). It deserves further study whether a small molecule or a protein, which is induced by JA, acts in suppression of the transcription of *GA20ox*.

GA signaling is negatively regulated by DELLA proteins and a growing body of evidence has shown that DELLAs are directly or indirectly involved in various signaling

pathway: ABA, ethylene, JA, auxin, and cytokinin (Grant & Jones, 2009; Sun, 2011). After MeJA treatment, mutants deficient in DELLAs have partially decreased sensitivity to the induction of JA-inducible genes and in a constitutively active dominant DELLA mutant JA-responsive gene induction is enhanced (Navarro *et al.*, 2008), since DELLAs directly bind JAZ proteins (suppressors of JA responses) and prevent JAZs from binding to MYC2 transcription factors (Hou *et al.*, 2010). Thus, through DELLA proteins, GAs represses JA signaling. Here we provide genetic evidence that JA signaling also antagonizes GA biosynthesis by mainly suppressing transcript accumulation of *GA20ox*. It is conceivable that JA-induced inhibition of stem elongation is also at least partly mediated DELLA proteins. JA interacts with auxin: JA promotes biosynthesis of auxin (Dombrecht *et al.*, 2007; Sun, JQ *et al.*, 2011) and affect auxin distribution and signaling (Chen *et al.*, 2011; Sun, JQ *et al.*, 2011). It is unclear whether JA cross-talks with other major hormones that are involved in growth regulation, such as BRs and cytokinin; however, given the increasing number of studies that have revealed cross-talks among various hormones, it is very likely that the growth phenotype irCDPK4/5 is not only due to the suppression effect of JA on GA biosynthesis but also JA-induced abnormal levels of other hormones.

Material and methods

Plant material

The silenced CDPK4/5 *Nicotiana attenuata* plants as well as the Na*CDPK4Pro:GUS* and Na*CDPK5Pro:GUS* plants were used from Yang *et al.* (in review). Seeds were originally collected in Utah (USA) and inbred for 31 generations in the glasshouse. Seed germination and plant cultivation followed Krügel *et al.* (2002). Crossing irMYB8, irAOC, irCOI1 and ovJMT with irCDPK4/5 plants was done by removing anthers before pollen maturation and hand-pollinate the stigmas with irCDPK4/5 pollen.

Histochemical GUS (β-glucuronidase) assays

Histochemical assays were done following Jefferson *et al*. (1987). Stem samples were cut and fixed in ice-cold 90% acetone for 2 h samples. Samples were then washed with ddH_2O and immersed in the enzymatic reaction mixture (1 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 2 mM ferricyanide, and 0.5 mM of ferrocyanide in 100 mM phosphate buffer, pH 7.4). The reaction was performed at 37 ^{o}C in the dark for 5 min to 30 min, and then the samples were cleared with pure ethanol. Pictures were taken with a microscope (Zeiss, Discovery. V8). For normal staining we used toluidine blue staining (Obrien *et al.*, 1964). Cell size was calculated using ImageJ *(rsbweb.nih.gov/ij/).* Scanning

electron microscopy was carried out in the laboratory of Dr. Martin Westermann at the Friedrich-Schiller-University in Jena (Germany). Stem were harvested and immediately frozen in liquid nitrogen and then dehydrated in a freeze dryer.

Plant treatment

For complementation assays plants were sprayed with 3 µM GA_3 for 21 days after being transferred to the glasshouse. For mimicking high JA levels, plants were treated with MeJA (150 µg/ 20 µl lanolin paste).

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted from ground samples using TRIzol reagent (Invitrogen) following the manufacturer's instructions. For qPCR analysis, 5 replicated biological samples were used. 0.5 µg of total RNA sample were reverse-transcribed using oligo$(dT)_{18}$ and Superscript II reverse transcriptase (Invitrogen). qPCR was performed on an a Mx3005P qPCR system (Stratagene, Santa Clara, CA, USA, http://www.stratagene.com) and qPCR Core Kit for SYBR® Green I (Eurogentec, Seraing, Belgium, http://www.eurogentec.com). Transcript levels were quantified normalized to *N. attenuata* elongation factor 1a (EF1a). All primer sequences used for qRT-PCR are listed in Table S1A. GenBank accession numbers for all sequences can be found in Table S1B.

Phytohormone analysis

100 mg of frozen plant material were homogenized in 2-mL microcentrifuge tubes containing 2 metal balls and 1 mL of ethyl acetate spiked with 200 ng of D_2-JA and $^{13}C_6$-JA-Ile. Homogenization was done twice with 200 strokes/min for 1 min using a Geno/Grinder 2000 (SPEX CertiPrep, Metuchen, NJ, USA). Samples were centrifuged at 13,000 g for 20 min at 4 °C. Supernatants were dried using a vacuum concentrator (Eppendorf AG, Hamburg, Germany). The residues were resuspended in 500 µL of 70% methanol by vortexing for 5 minutes, and centrifuged 10 minutes at 4 °C (13,000 g). Supernatants were transferred to crimp vials and sample measurements were carried out as described in Wu *et al*. (2007).

Analysis of secondary metabolites

About 200 mg of frozen plant tissue was homogenized and then extracted with 1 mL of 80% Methanol with 0.05% acetic acid. Samples were measured using a HPLC method described in Keinanen *et al.* (2001).

Purification and identification of N1-caffeoyl-N3-dihydrocaffeoylspermidine

Purification and NMD structural elucidation of N1-caffeoyl-N3-dihydrocaffeoylspermidine is described in Supplemental Method 1.

Molecular cloning and virus-induced gene silencing (VIGS)

Partial *GA20ox* (GenBank accession numbers: JQ413251) sequence was amplified using plasmid as templates and gene-specific primers (listed in Table S2). The PCR products were digested with appropriate restriction endonucleases and were further ligated into pTV00 to obtain the constructs pTV-GA20ox.

Agrobacterium tumefaciens carrying these constructs was inoculated into *N. attenuata* WT or irCDPK4/5 plants to obtain gene-silenced plants following a procedure optimized for *N. attenuata* (Saedler and Baldwin, 2004). Plants inoculated with *A. tumefaciens* carrying pTV00 (empty vector) were used for comparisons (EV plants). Plants silenced in *PDS* (*phytoene desaturase*) were used to monitor the degree of VIGS, since these plants showed a photo-bleaching phenotype (Saedler and Baldwin, 2004). About 14 days after inoculation, when the leaves of *PDS*-silenced plants were completely white, growth measurements were performed.

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Supporting Information

Supplemental Figure 1. *CDPK4* and *CDPK5* expression in *N. attenuata* during stem growth.

Supplemental Figure 2. Jasmonic acid levels in leaves of wild-type and irCDPK4/5 plants.

Supplemental Figure 3. High density of phenolic compounds in irCDPK5.

Supplemental Figure 4. HPLC chromatograms of the secondary metabolites of wild-type, irCDPK4/5, irCDPK4/5×irCOI1 and irCOI1 stem tissues.

Supplemental Figure 5. Secondary metabolites in leaf tissues of wild-type and irCDPK4/5.

Supplemental Figure 6. Contents of secondary metabolites.

Supplemental Figure 7. Plant heights are correlated with JA contents.

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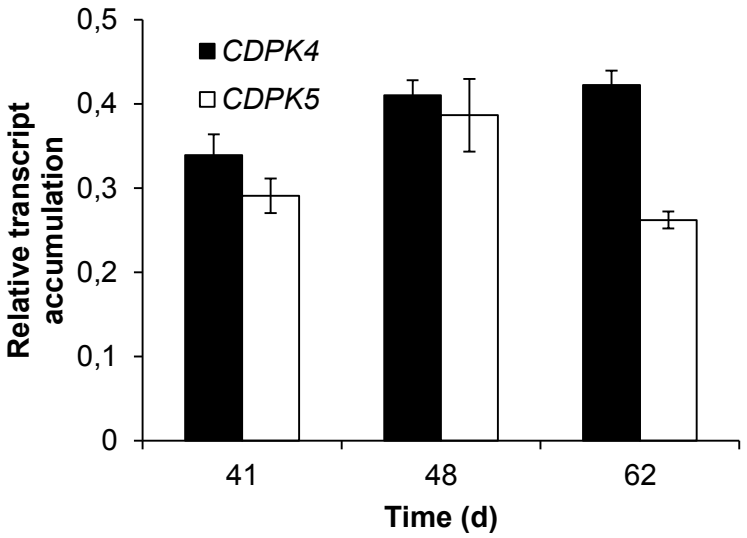
Supplemental table S1A. Primer used for qRT-PCR

Supplemental table S1B. GenBank accession numbers

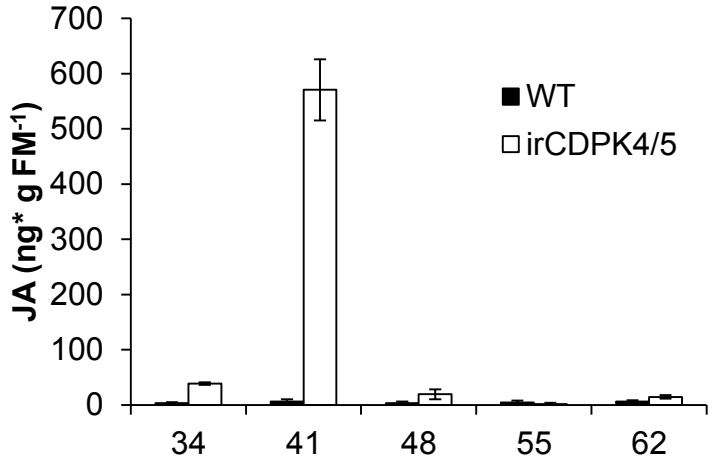
Supplemental table S2. Primer used to clone *NaGA20ox* into PTV00 to obtain VIGS construct.

Supplemental Material



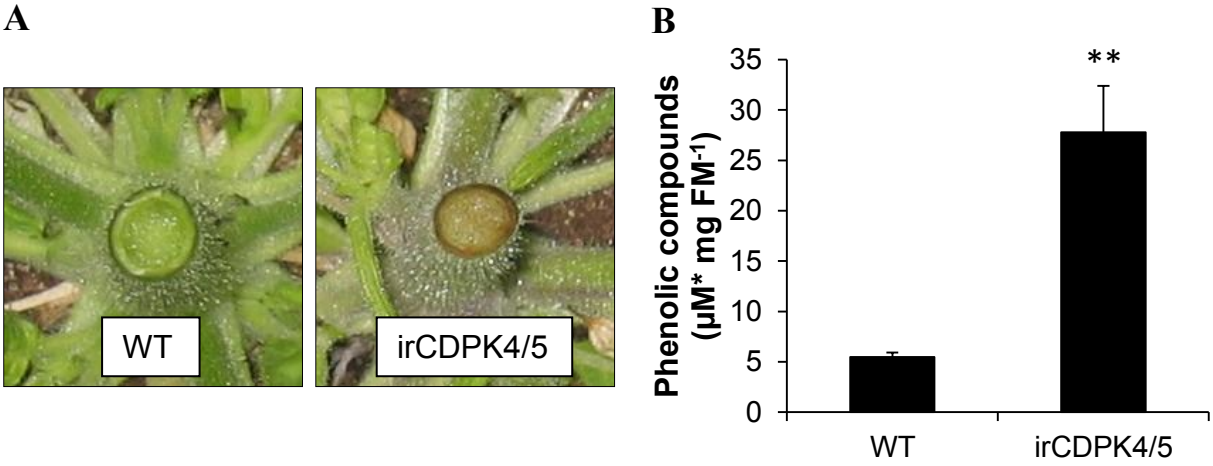
Supplemental Figure 1. *CDPK4* and *CDPK5* expression in *N. attenuata* during stem growth.

Transcript levels (mean ± SE) of *CDPK4* and *CDPK5* were measured in stems of wild-type *N. attenuata* using qRT-PCR (N = 5).



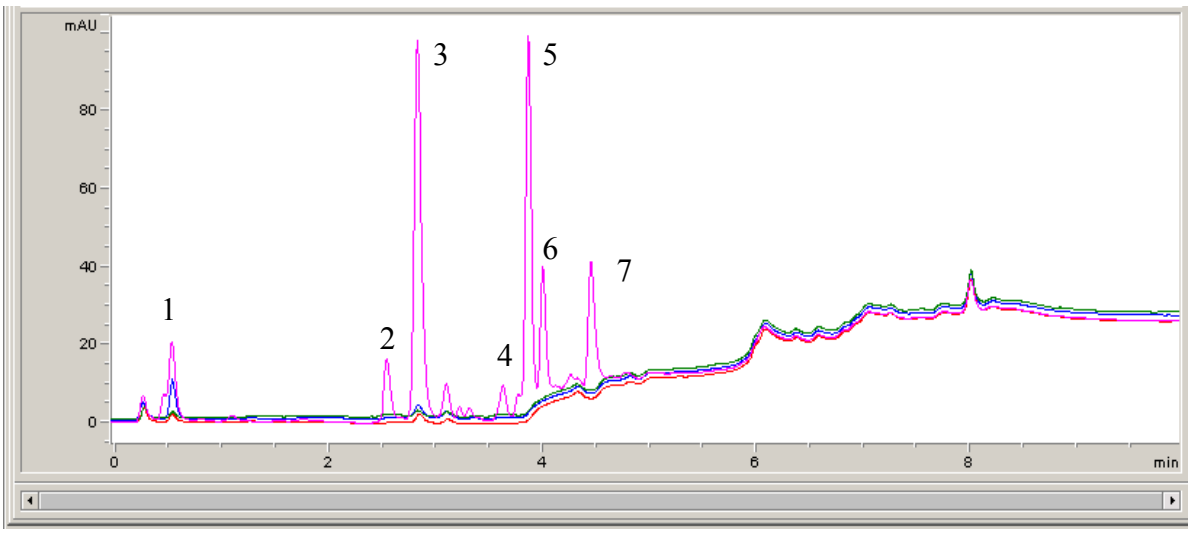
Supplemental Figure 2. Jasmonic acid levels in leaves of wild-type and irCDPK4/5 plants.

Wild-type (WT) and irCDPK4/5 plants were cultivated concurrently. The contents of JA (mean ± SE) were measured in the leaves until 62 days after germination when plants almost completely reproduction (N = 5).



Supplemental Figure 3. High density of phenolic compounds in irCDPK5.

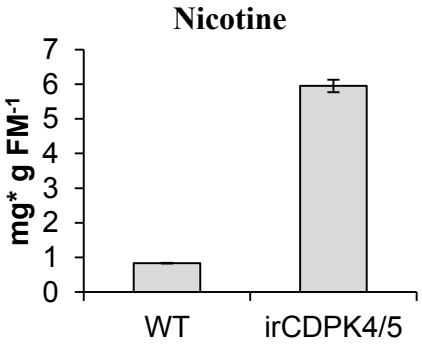
(A) Photographs of wild-type (WT) and irCDPK4/5 stems, 5 min after and exposure to air. (B) irCDPK4/5 stem tissue shows a higher amount of total phenolic compounds (mean ± SE). Asterisks indicate significant differences between WT and irCDPK4/5 plants (*t*-test; *, $P < 0.05$; **, $P < 0.01$; $N = 5$).

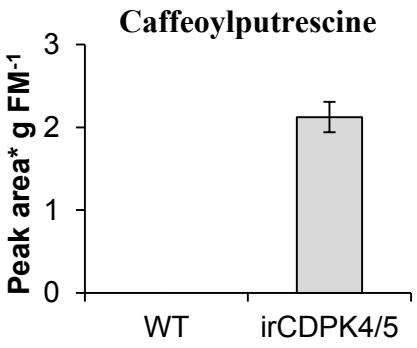


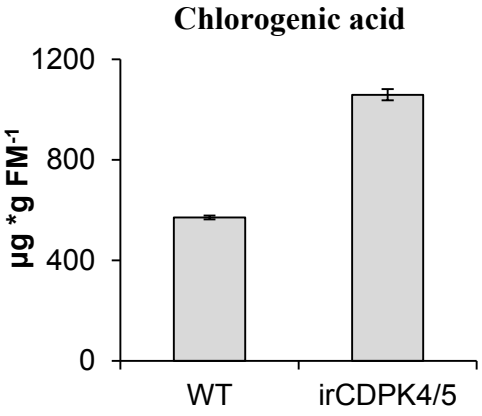
Peak Nr.	Tetention time (min)	Compound
1	0.53	Nicotine
2	2.45	Caffeoylputrescine
3	2.77	Chlorogenic acid
4	2.95	Cryptochlorogenic acid
5	3.97	Dicaffeoyl spermidine
6	4.1	N1-caffeoyl-N3-Dihydrocaffeoylspermidine
7	4.62	Rutin

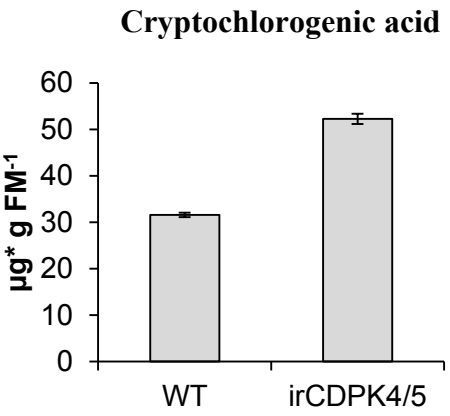
Supplemental Figure 4. HPLC chromatograms of the secondary metabolites of wild-type, irCDPK4/5, irCDPK4/5×irCOI1 and irCOI1 stem tissues.

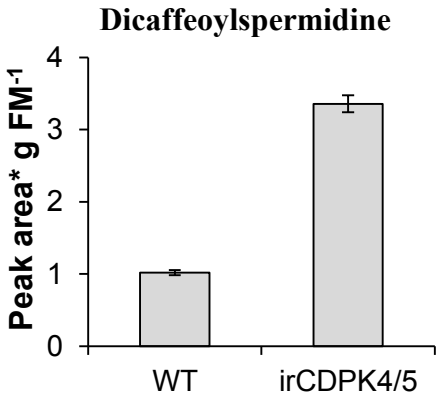
Secondary metabolites in wild-type (WT), irCDPK4/5, irCDPK4/5×irCOI1 and irCOI1 stem tissues were separated on a HPLC and the chromatographs were overlaid. WT: blue line; irCDPK4/5: pink line; irCDPK4/5×irCOI1: green line; irCOI1: red line.

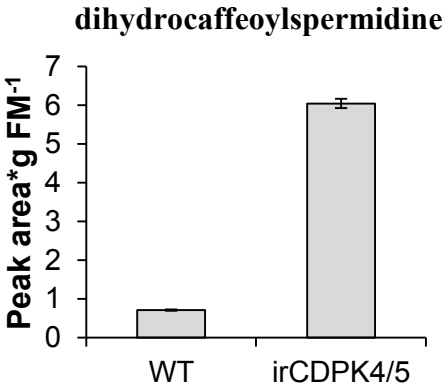

Nicotine
mg* g FM-1
0
1
2
3
4
5
6
7
WT
irCDPK4/5

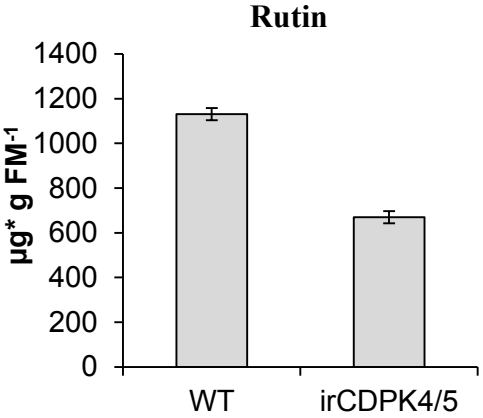

Caffeoylputrescine
Peak area* g FM-1
0
1
2
3
WT
irCDPK4/5


Chlorogenic acid
µg *g FM-1
0
400
800
1200
WT
irCDPK4/5


Cryptochlorogenic acid
µg* g FM-1
0
10
20
30
40
50
60
WT
irCDPK4/5

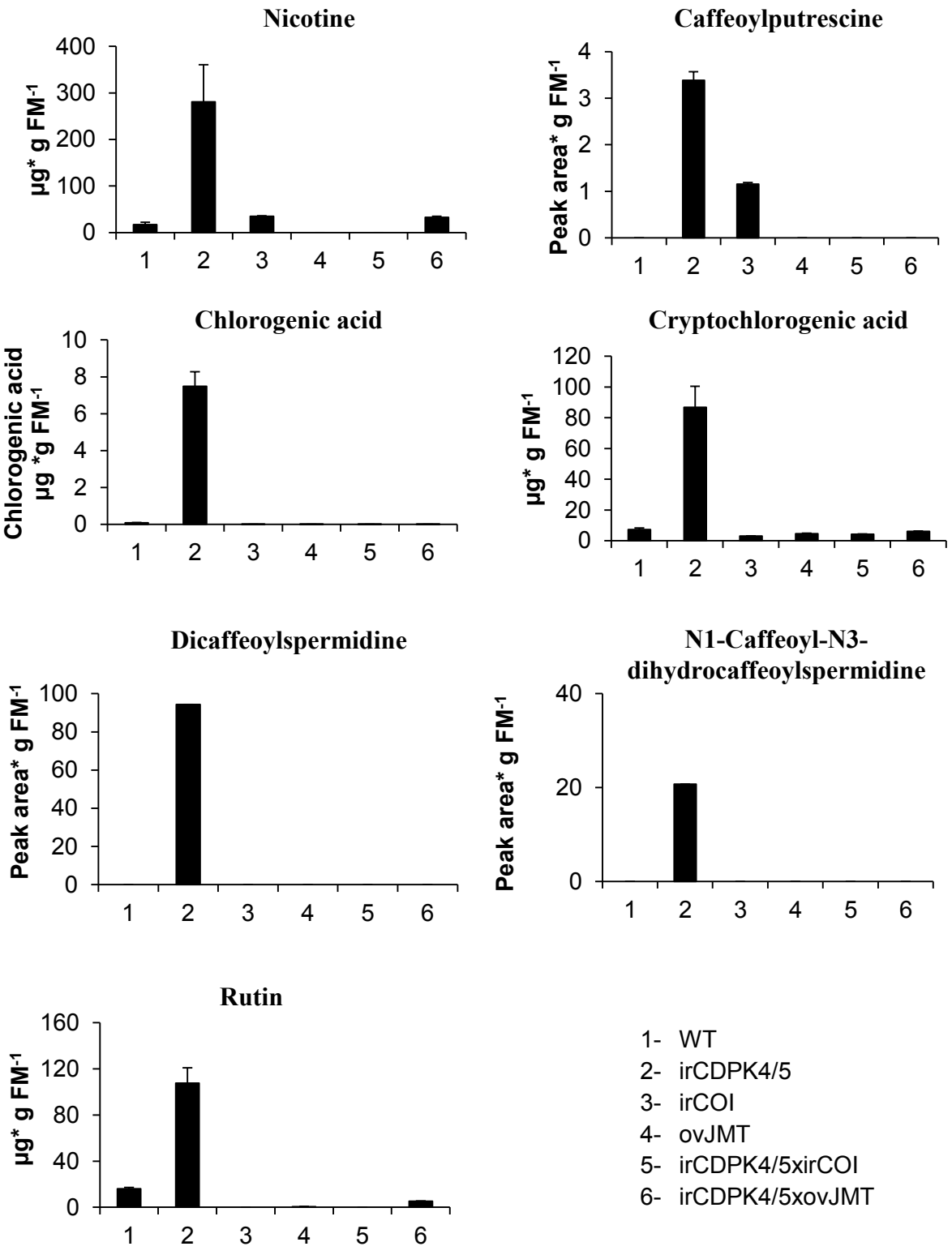

Dicaffeoylspermidine
Peak area* g FM-1
0
1
2
3
4
WT
irCDPK4/5


N1-Caffeoyl-N3-dihydrocaffeoylspermidine
Peak area*g FM-1
0
1
2
3
4
5
6
7
WT
irCDPK4/5


Rutin
µg* g FM-1
0
200
400
600
800
1000
1200
1400
WT
irCDPK4/5

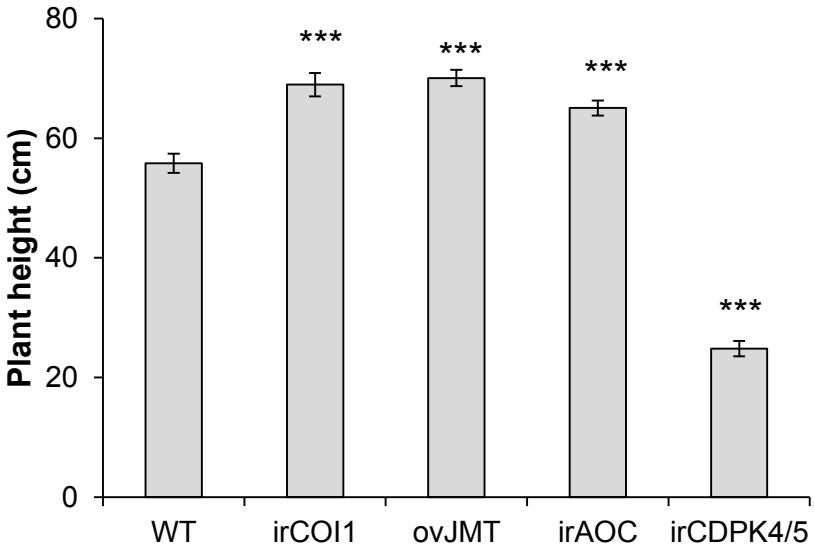
Supplemental Figure 5. Secondary metabolites in leaf tissues of wild-type and irCDPK4/5.

The contents (mean ± SE) of nicotine, caffeoylputrescine, chlorogenic acid, cryptochlorogenic acid, dicaffeoylspermidine, N1-caffeoyl-N3-dihydrocaffeoylspermidine, and rutin were measured in the leaf tissues of 48 days old Wild-type (WT) and irCDPK4/5. All data were obtained from five biological replicates.


Nicotine
µg* g FM-1
400
300
200
100
0
1 2 3 4 5 6
Caffeoylputrescine
Peak area* g FM-1
4
3
2
1
0
1 2 3 4 5 6
Chlorogenic acid
Chlorogenic acid µg *g FM-1
10
8
6
4
2
0
1 2 3 4 5 6
Cryptochlorogenic acid
µg* g FM-1
120
100
80
60
40
20
0
1 2 3 4 5 6
Dicaffeoylspermidine
Peak area* g FM-1
100
80
60
40
20
0
1 2 3 4 5 6
N1-Caffeoyl-N3-dihydrocaffeoylspermidine
Peak area* g FM-1
40
20
0
1 2 3 4 5 6
Rutin
µg* g FM-1
160
120
80
40
0
1 2 3 4 5 6
1- WT
2- irCDPK4/5
3- irCOI
4- ovJMT
5- irCDPK4/5xirCOI
6- irCDPK4/5xovJMT

Supplemental Figure 6. Contents of secondary metabolites.

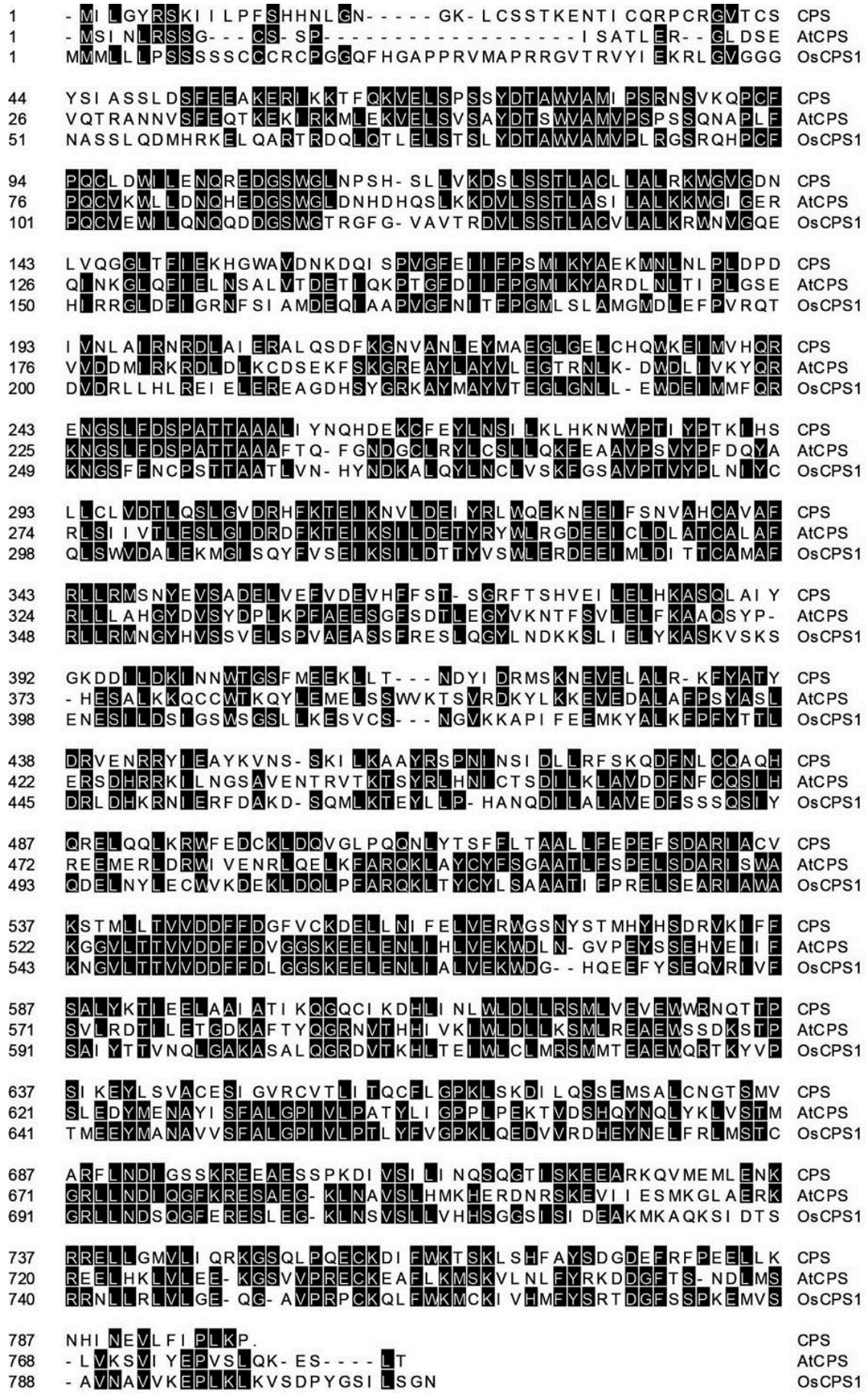
Wild-type (WT), irCDPK4/5, irCOI1, ovJMT, irCDPK4/5×irCOI1, and irCDPK4/5×ovJMT plants were cultivated concurrently. The contents (mean ± SE) of nicotine, caffeoylputrescine, chlorogenic acid, cryptochlorogenic acid, dicaffeoylspermidine, N1-caffeoyl-N3-dihydrocaffeoylspermidine, and rutin were measured in the stems tissues (48 days old plants). All data were obtained from five biological replicates.



Supplemental Figure 7. Plant heights are correlated with JA contents.

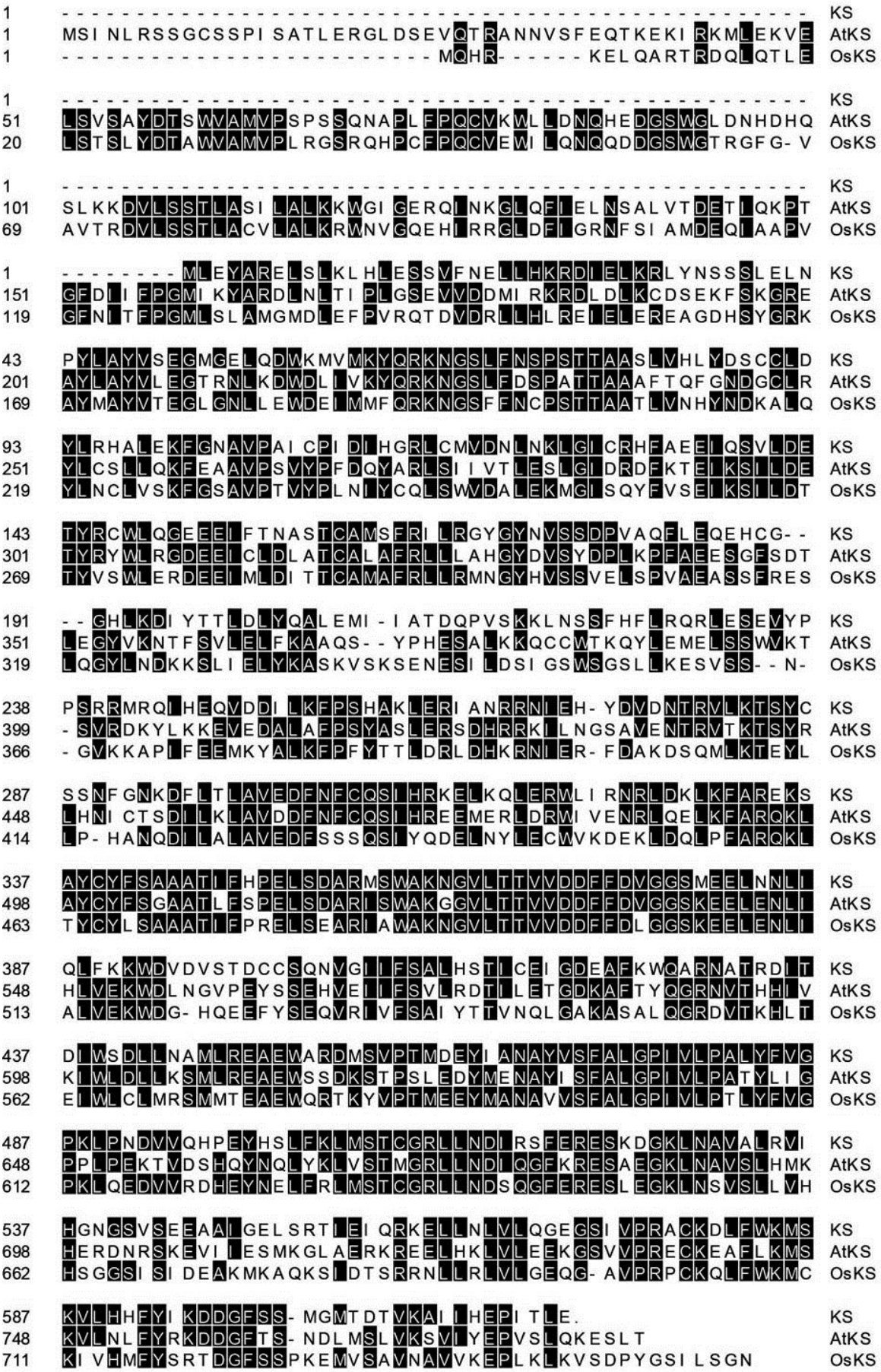
Wild-type, irCOI1, ovJMT, irAOC, and irCDPK4/5 plants were grown concurrently. The plant heights (mean ± SE) were measured in 40 days old plants. Asterisks indicate significant differences between WT and other types of plants (*t*-test; ***, $P < 0.001$; $N = 5$).

A



Shade (with solid black) residues that match the Consensus exactly.

B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1 ----------MQHR------KELQARTRDQLQTLE OsKS
1 ---------- KS
51 LSVSAYDTSWVAMVPSPSSQNAPLFPQCVKWLLDNQHEDGSWGLDNHDHQ AtKS
20 LSTSLYDTAWVAMVPLRGSRQHPCFPQCVEWILQNQQDDGSWGTRGFG-V OsKS
1 ---------- KS
101 SLKKDVLSSTLASILALKKWGIGERQINKGLQFIELNSALVTDETIQKPT AtKS
69 AVTRDVLSSTLACVLALKRWNVGQEHIRRGLDFIGRNFSIAMDEQIAAPV OsKS
1 ---------MLEYARELSLKLHLESSVFNELLHKRDIELKRLYNSSSLELN KS
151 GFDIIFPGMIKYARDLNLTIPLGSEVVDDMIRKRDLDLKCDSEKFSKGRE AtKS
119 GFNITFPGMLSLAMGMDLEFPVRQTDVDRLLHLREIELEREAGDHSYGRK OsKS
43 PYLAYVSEGMGELQDWKMVMKYQRKNGSLFNSPSTTAASLVHLYDSCCLD KS
201 AYLAYVLEGTRNLKDWDLIVKYQRKNGSLFDSPATTAAAFTQFGNDGCLR AtKS
169 AYMAYVTEGLGNLLEWDEIMMFQRKNGSFFNCPSTTAATLVNHYNDKALQ OsKS
93 YLRHALEKFGNAVPAICPIDIHGRLCMVDNLNKLGICRHFAEEIQSVLDE KS
251 YLCSLLQKFEAAVPSVYPFDQYARLSIIVTLESLGIDRDFKTEIKSILDE AtKS
219 YLNCLVSKFGSAVPTVYPLNIYCQLSWVDALEKMGISQYFVSEIKSILDT OsKS
143 TYRCWLQGEEEIFTNASTCAMSFRILRGYGYNVSSDPVAQFLEQEHCG-- KS
301 TYRYWLRGDEEICLDLATCALAFRLLLAHGYDVSYDPLKPFAEESGFSDT AtKS
269 TYVSWLERDEEIMLDITTCAMAFRLLRMNGYHVSSVELSPVAEASSFRES OsKS
191 --GHLKDIYTTLDLYQALEMI-IATDQPVSKKLNSSFHFLRQRLESEVYP KS
351 LEGYVKNTFSVLELFKAAQS--YPHESALKKQCCWTKQYLEMELSSWVKT AtKS
319 LQGYLNDKKSLIELYKASKVSKSENESILDSIGSWSGSLLKESVSS--N- OsKS
238 PSRRMRQIHEQVDDILKFPSHAKLERIANRRNIEH-YDVDNTRVLKTSYC KS
399 -SVRDKYLKKEVEDALAFPSYASLERSDHRRKILNGSAVENTRVTKTSYR AtKS
366 -GVKKAPIFEEMKYALKFPFYTTLDRLDHKRNIER-FDAKDSQMLKTEYL OsKS
287 SSNFGNKDFLTLAVEDFNFCQSIHRKELKQLERWLIRNRLDKLKFAREKS KS
448 LHNICTSDILKLAVDDFNFCQSIHREEMERLDRWIVENRLQELKFARQKL AtKS
414 LP-HANQDILALAVEDFSSSQSIYQDELNYLECWVKDEKLDQLPFARQKL OsKS
337 AYCYFSAAATIFHPELSDARMSWAKNGVLTTVVDDFFDVGGSMEELNNLI KS
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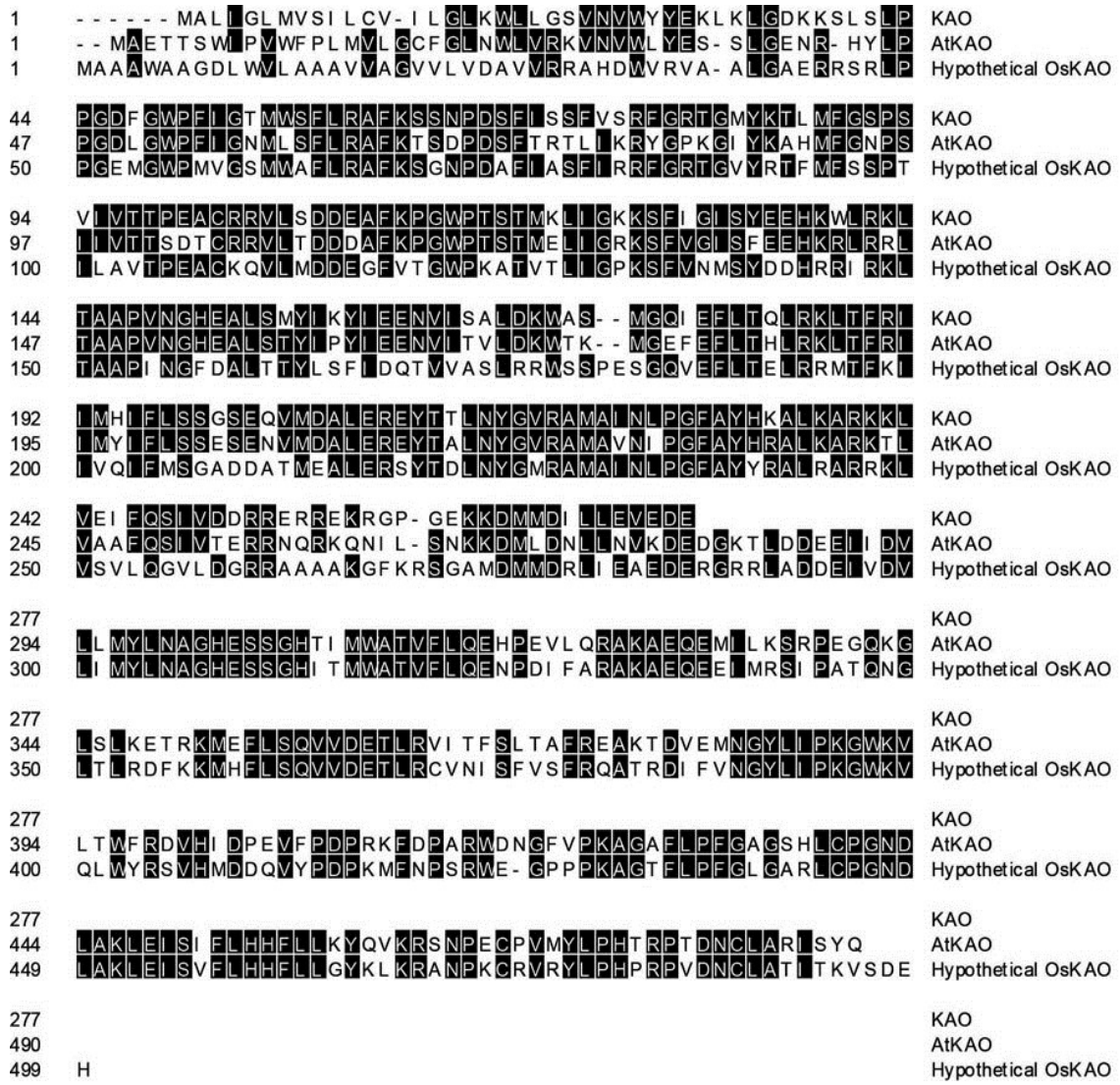
463 TYCYLSAAATIFPRELSEARIAWAKNGVLTTVVDDFFDLGGSKEELENLI OsKS
387 QLFKKWDVDVSTDCCSQNVGIIFSALHSTICEIGDEAFKWQARNATRDIT KS
548 HLVEKWDLNGVPEYSSEHVEIIFSVLRDTILETGDKAFTYQGRNVTHHIV AtKS
513 ALVEKWDG-HQEEFYSEQVRIVFSAIYTTVNQLGAKASALQGRDVTKHLT OsKS
437 DIWSDLLNAMLREAEWARDMSVPTMDEYIANAYVSFALGPIVLPALYFVG KS
598 KIWLDLLKSMLREAEWSSDKSTPSLEDYMENAYISFALGPIVLPATYLIG AtKS
562 EIWLCLMRSMMTEAEWQRTKYVPTMEEYMANAVVSFALGPIVLPTLYFVG OsKS
487 PKLPNDVVQHPEYHSLFKLMSTCGRLLNDIRSFERESKDGKLNAVALRVI KS
648 PPLPEKTVDSHQYNQLYKLVSTMGRLLNDIQGFKRESAEGKLNAVSLHMK AtKS
612 PKLQEDVVRDHEYNELFRLMSTCGRLLNDSQGFERESLEGKLNSVSLLVH OsKS
537 HGNGSVSEEAAIGELSRTIEIQRKELLNLVLQGEGSIVPRACKDLFWKMS KS
698 HERDNRSKEVIIESMKGLAERKREELHKLVLEEKGSVVPRECKEAFLKMS AtKS
662 HSGGSISIDEAKMKAQKSIDTSRRNLLRLVLGEQG-AVPRPCKQLFWKMC OsKS
587 KVLHHFYIKDDGFSS-MGMTDTVKAILHEPITLE. KS
748 KVLNLFYRKDDGFTS-NDLMSLVKSVIYEPVSLQKESLT AtKS
711 KIVHMFYSRTDGFSSPKEMVSAVNAVVKEPLKLKVSDPYGSILSGN OsKS

C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1 MAAAWAAGDLWVLAAAVVAGVVLVDAVVRRAHDWVRVA-ALGAERRSRLP Hypothetical OsKAO
44 PGDFGWPFIGTMWSFLRAFKSSNPDSFISSFVSRFGRTGMYKTLMFGSPS KAO
47 PGDLGWPFIGNMLSFLRAFKTSDPDSFTRTLIKRYGPKGIYKAHMFGNPS AtKAO
50 PGEMGWPMVGSMWAFLRAFKSGNPDAFIASFIRRFGRTGVYRTFMFSSPT Hypothetical OsKAO
94 VIVTTPEACRRVLSDDEAFKPGWPTSTMKLIGKKSFIGISYEEHKWLRKL KAO
97 IIVTTSDTCRRVLTDDDAFKPGWPTSTMELIGRKSFVGISFEEHKRLRRL AtKAO
100 ILAVTPEACKQVLMDDEGFVTGWPKATVTLIGPKSFVNMSYDDHRRIRKL Hypothetical OsKAO
144 TAAPVNGHEALSMYIKYIEENVISALDKWAS--MGQIEFLTQLRKLTFRI KAO
147 TAAPVNGHEALSTYIPYIEENVITVLDKWTK--MGEFEFLTHLRKLTFRI AtKAO
150 TAAPINGFDALTTYLSFIDQTVVASLRRWSSPESGQVEFLTELRRMTFKI Hypothetical OsKAO
192 IMHIFLSSGSEQVMDALEREYTTLNYGVRAMAINLPGFAYHKALKARKKL KAO
195 IMYIFLSSESENVMDALEREYTALNYGVRAMAVNIPGFAYHRALKARKTL AtKAO
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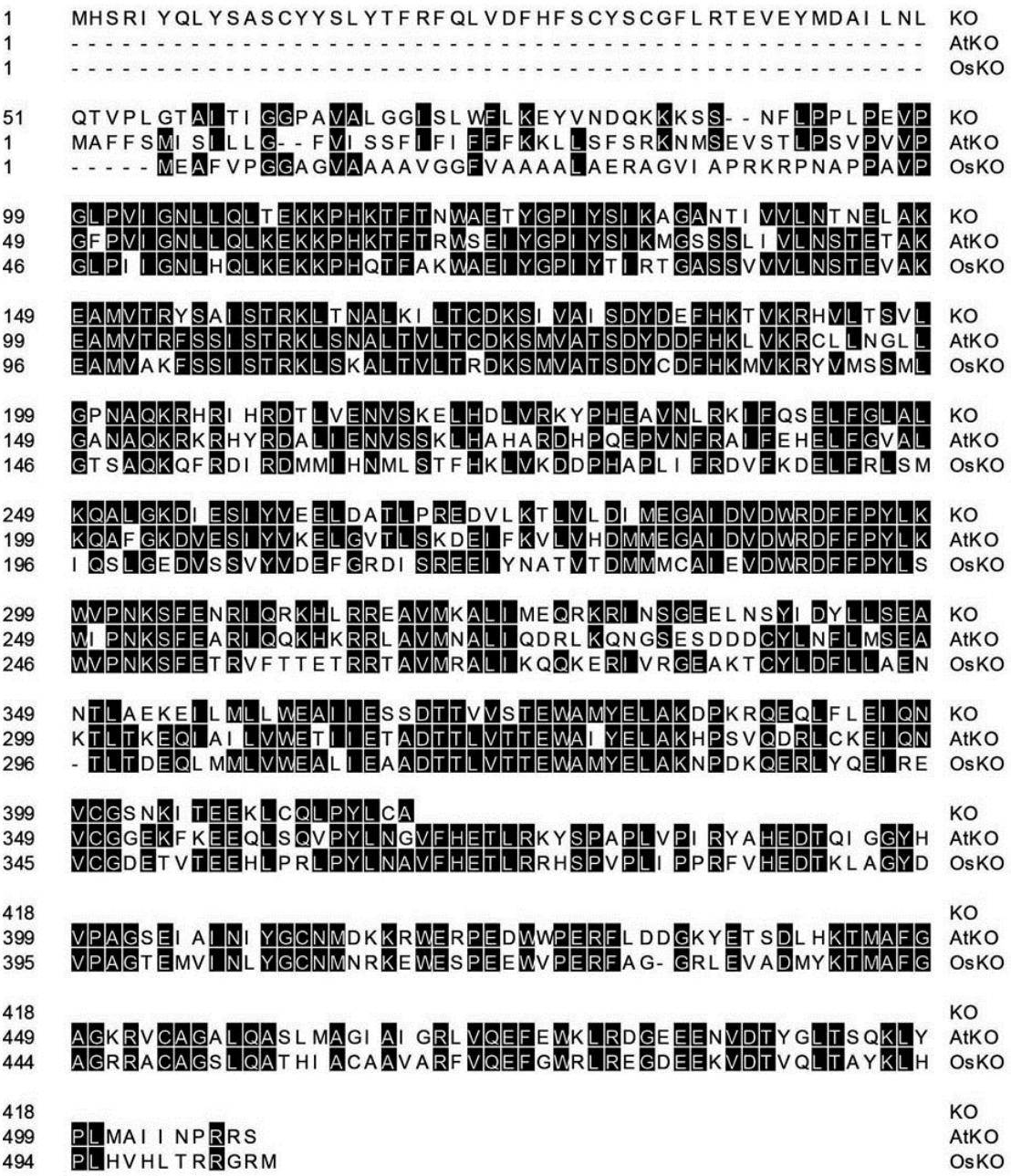
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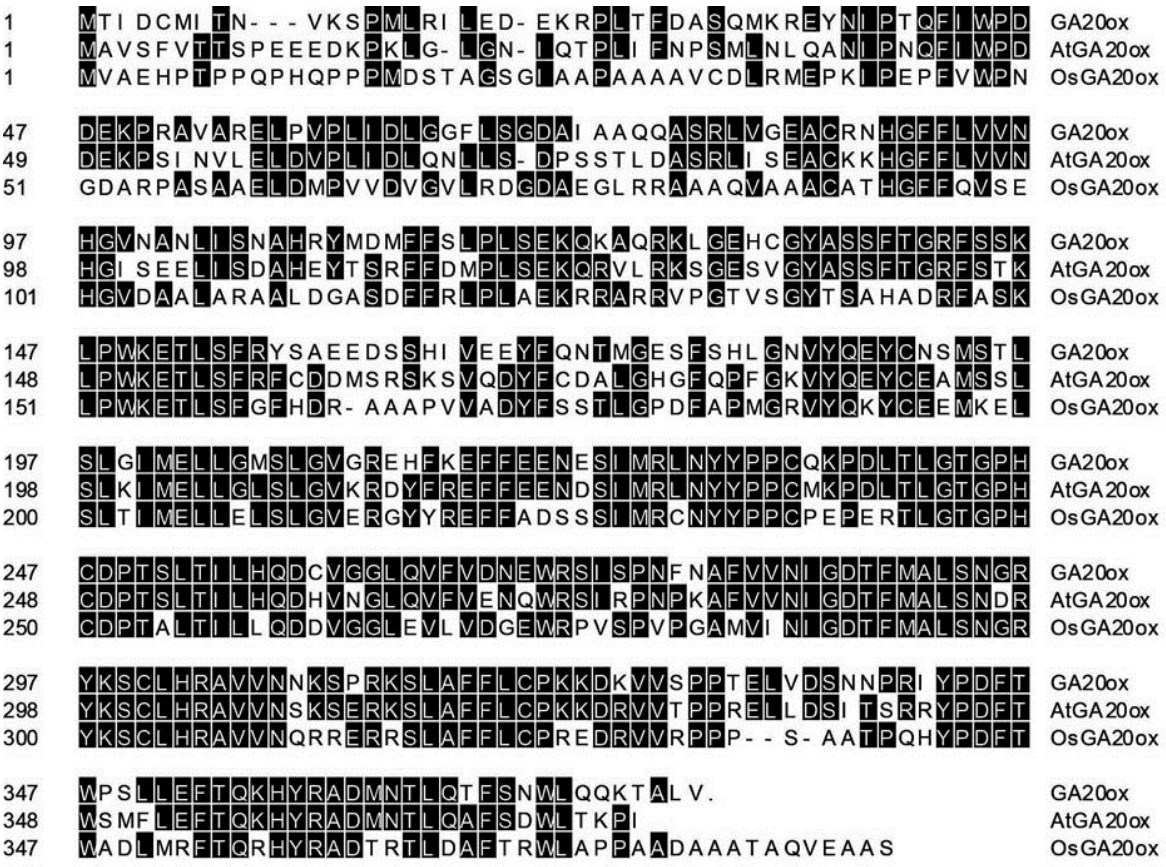
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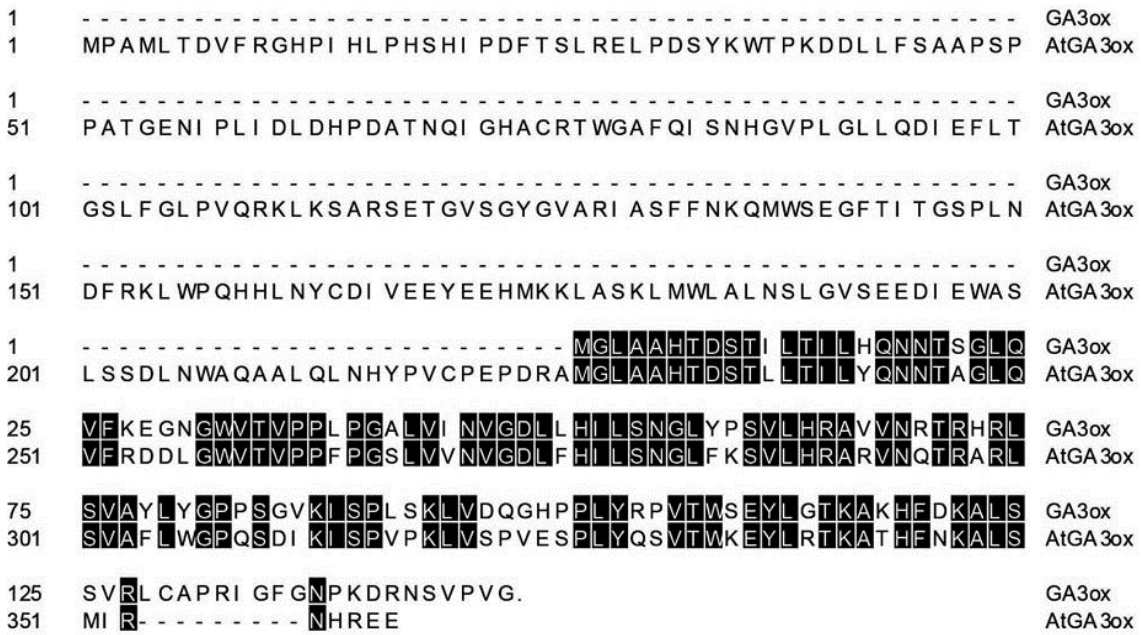
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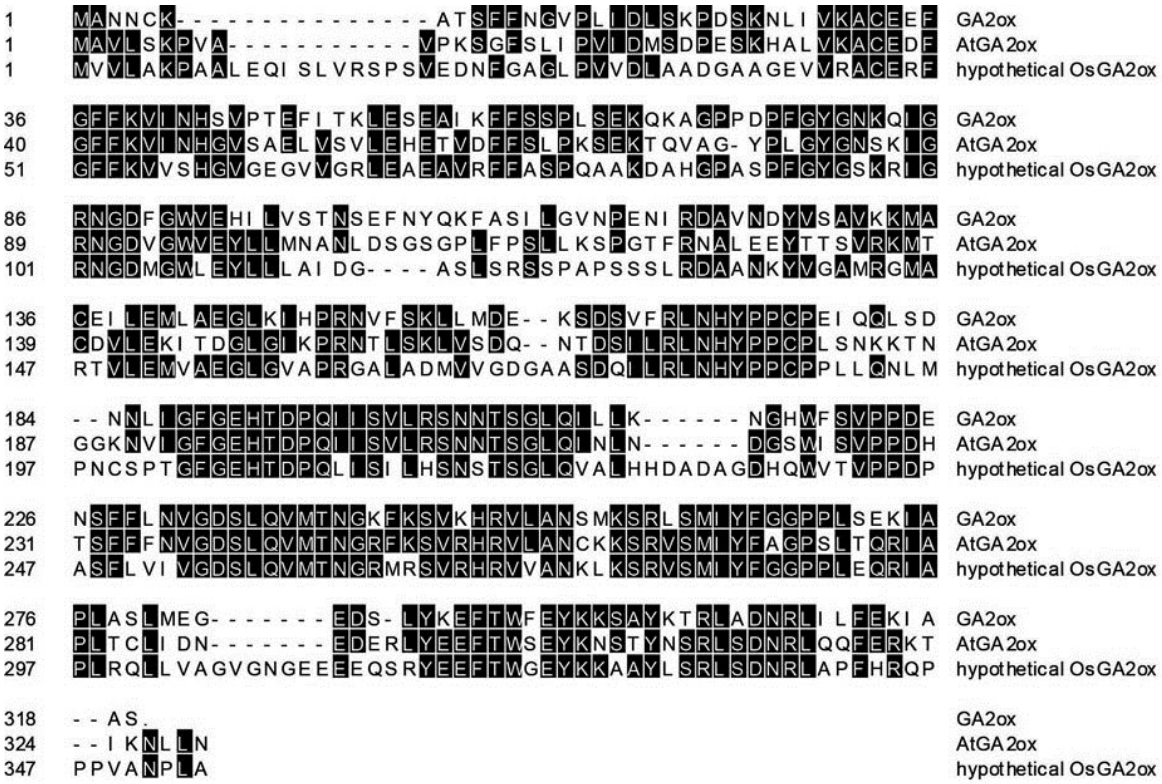
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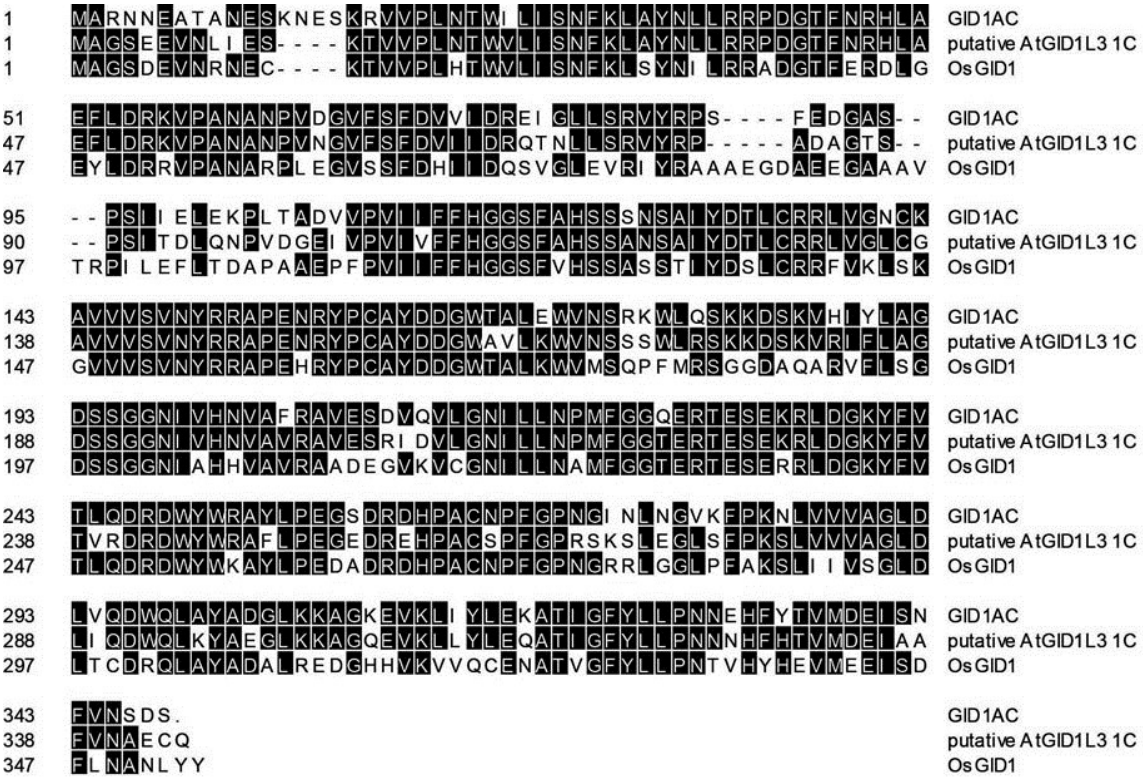
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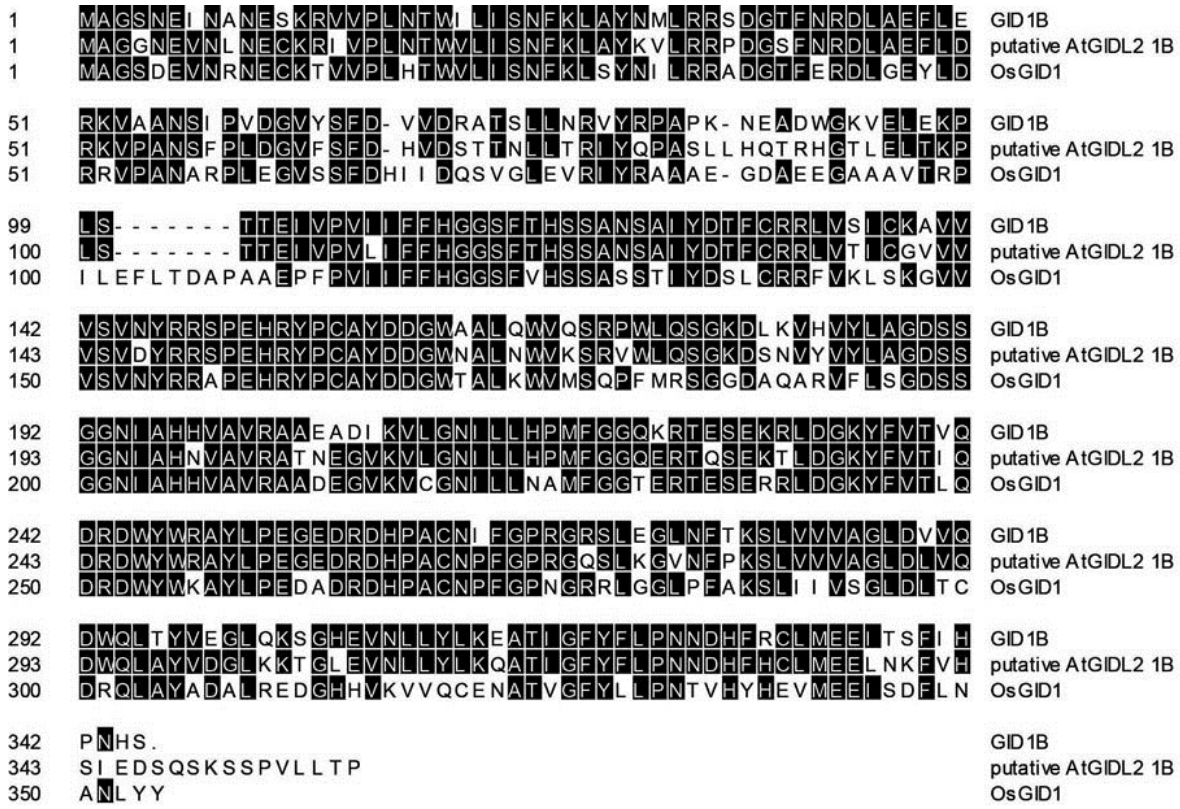
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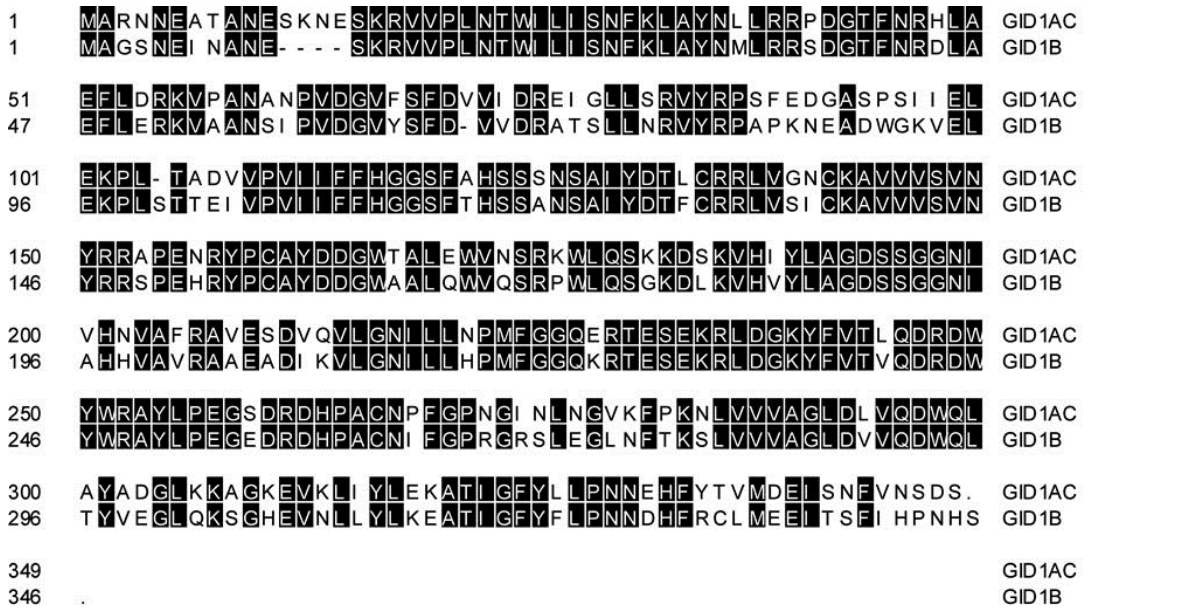
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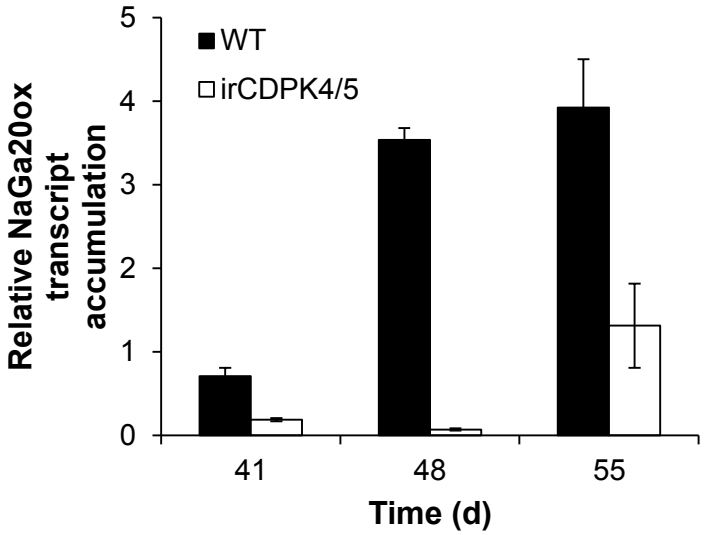
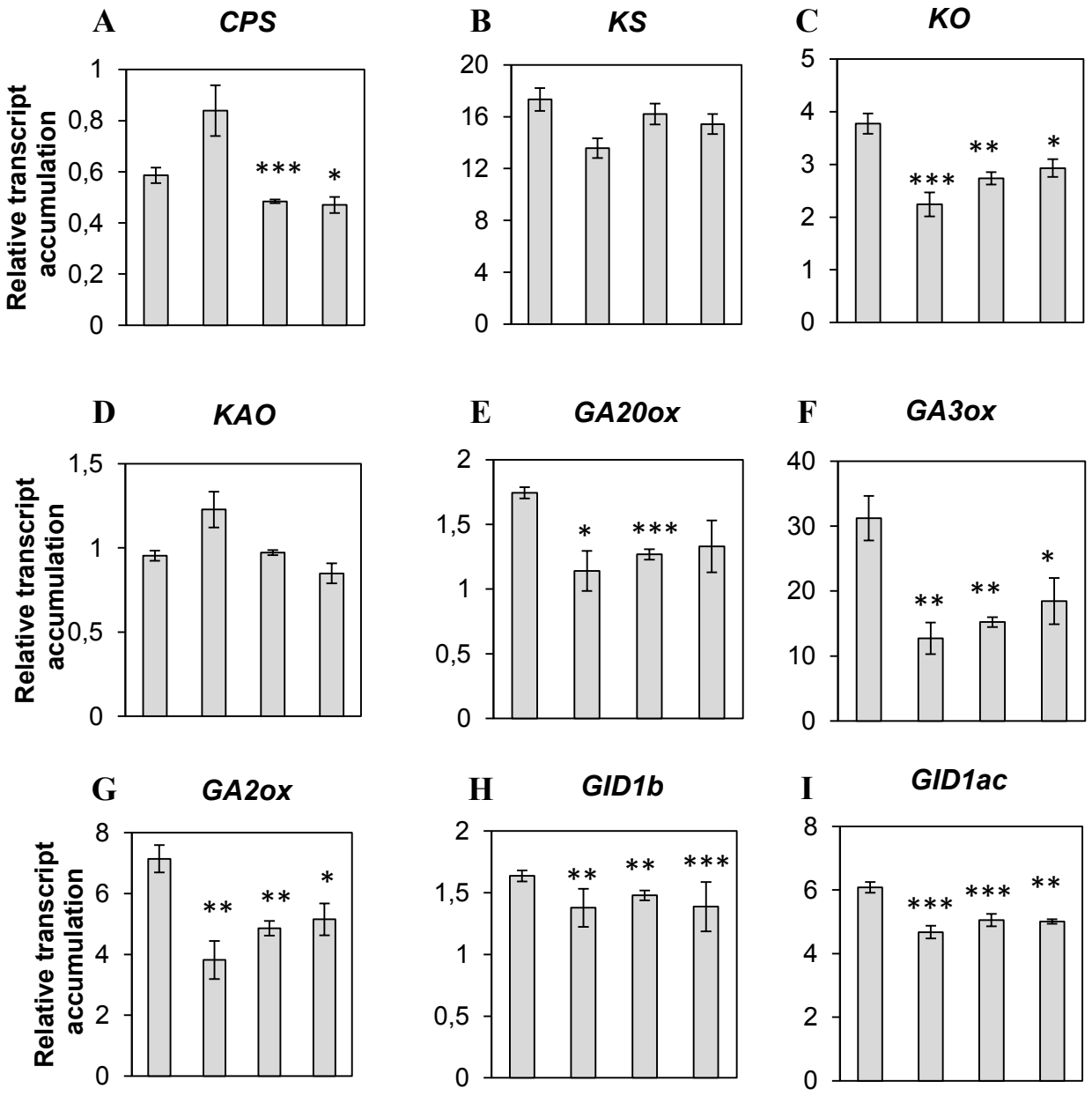
Supplemental Figure 8. Alignments of protein sequences.

Protein sequences of *N. attenuata* (A) CPS, (B) KS, (C) KO, (D) KAO, (E) GA20ox, (F) GA3ox, (G) GA2ox, (H) GID1B, and (I) GID1AC were aligned with respective homologues from *Arabidopsis thaliana* and *Oryza sativa* (indicated by the At and Os in front of protein names). (J) Alignment of *N. attenuata* GID1B and GID1AC.



Supplemental Figure 9. *GA20ox* expression in wild-type and irCDPK4/5 *N. attenuata* plants over time.

Wild-type (WT) and irCDPK4/5 plants were grown concurrently. The transcript levels (mean ± SE) of *GA20ox* were measured in stems at different days after germination (N = 5).



Supplemental Figure 10. Relative transcript accumulation of GA genes in leaves of WT, irCDPK4/5, irCDPK4/5xirAOC and irAOC plants.

Asterisks indicate significant differences between WT and other plants (*t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; $N = 5$).

Supplemental Methods 1. Isolation and identification of N1-caffeoyl-N3-dihydrocaffeoylspermidine

100 mg plant material was ground in liquid N_2 and extracted with 10 ml 80% MeOH in ultrasonic for 1h. The extract solution was filtered. MeOH in the filtrate was evaporated in vacuum under 40°C, then the residual aqueous solution was loaded on a LiChrolut RP-18 SPE cartridge (40-63 μm, 200mg, 3 ml. Merck KGaA, Darmstadt, Germany), which was conditioned with 2 ml MeOH and then equilibrated with 2 ml H_2O before use. After discarding the eluate of 1.5 ml H_2O, the eluate of 1.5 ml 80% MeOH aqueous solution was collected for further isolation. The extract was purified on an Agilent series HP1100 (binary pump G1312A, autosampler G1367A, diode array detector G1315A, 200–700 nm) (Agilent Technologies, Waldbronn, Germany) using a Purospher RP18e column (5 μm, 250 × 4.6 mm. Merck KGaA, Darmstadt, Germany). The flow rate was kept at 1.0 ml min^{-1}. The following linear gradient of H_2O (solvent A) containing 0.1% trifluoroacetic acid and MeOH (solvent B) was applied: 0 min: 25% B, 30 min: 50% B, 32 min: 95% B, 37 min: 95% B, and 40 min: 25% B, followed by a 5 min equilibration step. The injection volume was 8 μl, and the peak of retention time 16.6 min was collected from five injections. Dried sample was dissolved with 90 μl CD_3OD, and transformed into 2 mm NMR tube. 1 and 2D NMR spectra were recorded on a Bruker AV 500 NMR spectrometer (Bruker Biospin, Karlsruhe, Germany), operating at 500 MHz for ^1H and 125 MHz for ^{13}C. A TCI cryoprobe (5 mm) was used to measure spectra at 298 K. The residual solvent signals were used as chemical references.

NMD spectrum data of N1-caffeoyl-N3-dihydrocaffeoylspermidine:

N1-caffeoyl-N3-dihydrocaffeoylspermidine: ^1H NMR (CD_3OD, 500 MHz) δ 7.41 (1H, d, J = 15.7 Hz, H-3′), 7.01 (1H, d, J = 2.1 Hz, H-5′), 6.90 (1H, dd, J = 8.4, 2.1 Hz, H-9′), 6.77 (1H, d, J = 8.4 Hz, H-8′), 6.69 (1H, d, J = 8.1 Hz, H-8″), 6.65 (1H, d, J = 2.1 Hz, H-5″), 6.54 (1H, dd, J = 8.1, 2.1 Hz, H-9″), 6.37 (1H, d, J = 15.7 Hz, H-2′), 3.35 (1H, t, J = 6.6 Hz, H-2), 3.24 (1H, t, J = 6.3 Hz, H-9), 2.88 (1H, t, J = 7.3 Hz, H-5), 2.79 (1H, t, J = 7.2 Hz, H-3″), 2.64 (1H, t, J = 7.2 Hz, H-7), 2.50 (1H, t, J = 7.2 Hz, H-2″), 1.73 (1H, m, H-8), 1.68 (1H, m, H-4), 1.67 (1H, m, H-3); ^{13}C NMR (CD_3OD, 125 MHz) δ 176.6 (C-1″), 169.6 (C-1′), 149.0 (C-7′), 146.8 (C-6′), 146.2 (C-6″), 144.7 (C-7″), 142.5 (C-3′), 133.3 (C-4″), 128.1 (C-4′), 122.2 (C-9′), 120.7 (C-9″), 118.1 (C-2′), 116.8 (C-5″), 116.44 (C-8′), 116.40 (C-8″), 115.0 (C-5′), 48.6 (C-5), 45.9 (C-7), 39.5 (C-2), 38.4 (C-2″), 36.5 (C-9), 31.9 (C-3″), 27.6 (C-3, 8), 24.6 (C-4).

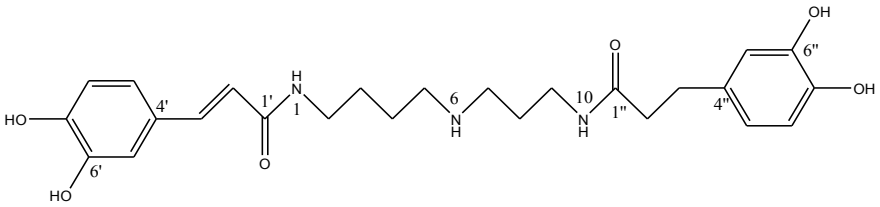


Figure structure of N1-caffeoyl-N3-dihydrocaffeoylspermidine

Supplemental table S1A. Primer used for qRT-PCR

Primer	Sequence (5'-3')
CPS forward	TCA AAG AGA AGA TGG ATC TTG GG
CPS reverse	AGG TGA AAT CTG ATC CTT GTT ATC
KS forward	GTC ATG GGC CAA GAA TGG TG
KS reverse	CGC TGA AAA TAT GAT GCC CAC
KO forward	ACG TAC TGA CCA GTG TTC TAG
KO reverse	ACTTCCTAACCAAATCATGCAGTT
KAO forward	GGT GAT GTT GAG TCT CTA CTG T
KAO reverse	CCA ACC TTT TGG AAT AGT ATA ACC
GA3ox forward	TTG CTG CAC ATA CGG ATT CTA C
GA3ox reverse	TAC GTT GAT AAC TAA TGC ACC CG
GA2ox forward	TGG CGT GTG AGA TTC TTG AAA TG
GA2ox reverse	ATT GAG CCT GAA AAC AGA GTC AC
GA20ox forward	TGC TGT GGC ACG TGA ACT TC
GA20ox reverse	CGTTAACTCCGTGGTTAACTAC
GID1B forward	GCATTTGCAAGGCTGTTGTTG
GID1B reverse	TGAAGCCATGGCCTCGATTG
GID1AC forward	GAT GGA GCT TCA CCG AGC AT
GID1AC reverse	CATAGCGTGTCATAGATAGCAC
EF1A forward	CCACACTTCCCACATTGCTG
EF1A reverse	CGCATGTCCCTCACAGCAAA

Supplemental table S1B. GenBank accession numbers

Gene name	GenBank accession number
NaCPS	JQ413245
NaKS	JQ413246
NaKAO	JQ413247
NaKO	JQ413248
NaGA2ox	JQ413249
NaGA3ox	JQ413250
NaGA20ox	JQ413251
NaGID1B	JQ413253
NaGID1AC	JQ413254

Supplemental table S2. Primer used to clone *NaGA20ox* into PTV00 to obtain VIGS construct.

Primer	Primer sequence (5'-3')
NaGA20ox-VIGS-*Hind*III	GCGGCG **GTCGAC** GCTCAAAGGAAACTTGGAGAG
NaGA20ox-VIGS-*Bam*HI	GCGGCG **GGATCC** GATAAACATTCCCAAGATGGCTA

Chapter 6: Discussion

Plants are challenged constantly by environmental changes and different stress factors. Thus they have evolved to have complex signaling networks, which modulate various aspects of plant physiology, to cope with unfavorable conditions to survive and reproduce.

Protein kinases are an important and large family of proteins that modulate developmental and adaptation processes in the plant kingdom. MAPKs play central roles in activation of plant responses against biotic and abiotic stresses (Bergmann *et al.*, 2004; Lukowitz *et al.*, 2004; Pedley & Martin, 2005; Wang *et al.*, 2007; Andreasson & Ellis, 2010; Rodriguez *et al.*, 2010). Calcium-dependent protein kinases (CDPKs) are another elemental group of protein kinases, which are involved in development and stress adaption (Yoon *et al.*, 1999; Romeis *et al.*, 2000; Romeis, 2001; Romeis *et al.*, 2001; Ivashuta *et al.*, 2005).

MAPKKs in herbivore resistance against *M. sexta* in *N. attenuata*

MAPK cascades are composed of three members, MAPK kinase kinases (MAPKKKs) activate MAPK kinases (MAPKKs) and in turn, they activate MAPKs. The genome of the model plant *Arabidopsis thaliana* encodes 20 MAPK genes and similar numbers are found in other plants whose full genome sequences are available (Hamel *et al.*, 2006). The MAPKK group consists of about 10 members in Arabidopsis and the MAPKKK group includes more than 60 members (MAPK Group, 2002). The relatively small number of MAPKKs suggests that they might have multiple MAPK targets and that interactions among different signaling pathways might be converged at the level of MAPKKs (MAPK Group., 2002; Hamel *et al.*, 2006; Andreasson & Ellis, 2010).

In *N. attenuata*, NaSIPK and NaWIPK are pivotal MAPKs that regulate plant responses to herbivory (Wu *et al.*, 2007). Another MAPK, NaMPK4 is known to be important in drought stress responses and stomata regulation as well as in pathogen defenses (Hettenhausen *et al.*, 2012). However, the upstream MAPKKs involved in herbivore resistance were unknown. In my work, five MAPKKs were studied for their functions in plant resistance to herbivores. First, a genetic approach was used to knock down the transcript levels of two MAPKK genes, NaMEK2 and NaMKK1, and it was found NaMEK2 is important in mediating *M. sexta* herbivory-induced defense responses, while NaMKK1 plays only a minor role (Heinrich *et al.*, 2011). After simulated herbivory, NaMEK2 and other MAPKKs, but not NaMKK1, are required for the activation of NaSIPK and NaWIPK, and

thus JA and ethylene biosynthesis. Using overexpression systems, a few studies have demonstrated that in Arabidopsis AtMKK4 and AtMKK5, close homologues of NaMEK2, phosphorylate AtMPK3 and AtMPK6, homologues of NaSIPK and NaWIPK (Asai *et al.*, 2002 346). Overexpression of another close homologue of NaMEK2 in tobacco, NtMEK2, activates NtSIPK and NtWIPK (Yang *et al.*, 2001; Zhang & Liu, 2001). Consistent with the AtMKK4/AtMKK5–AtMPK6/AtMPK3 and NtMEK2–NtSIPK/NtWIPK cascade, we identified NaMEK2 to be located upstream of NaSIPK and NaWIPK in the herbivory-induced signaling pathway using a knock-down approach (virus-induced gene silencing, VIGS).

Silencing NaMEK2 only reduced 50% of herbivory-induced NaSIPK activity levels, although qPCR analysis indicated that only 5% of NaMEK2 transcript levels were detected in NaMEK2-VIGS plants. This leads to the speculation that one or more other unknown MAPKKs might phosphorylate NaSIPK after *M. sexta* attack. On the other hand we cannot rule out NaMEK2 protein levels might not have been knocked down effectively, and were sufficient for NaSIPK phosphorylation. Although NaMKK1 was not important in NaSIPK and NaWIPK activation and in JA accumulation, it functions in regulation of TPI activity after herbivory. TPI is essential in the direct defense against herbivores in solanaceous species (Ryan, 1989; Haq *et al.*, 2004). Although TPI activity levels were decreased in both NaMKK1-VIGS and NaMEK2-VIGS plants, *M. sexta* larvae gained similar masses on these plants compared with those on EV plants. It is possible, that the decrease in TPI activity was not sufficient to weaken plant defense. Additionally, green leave volatiles (GLVs) are released from wounded leaves during insect feeding and these C6 compounds are thought to function as indirect defense, but also feeding stimulants or herbivore attractants (Dicke & van Loon, 2000; Meldau *et al.*, 2009; Allmann *et al.*, 2010). In *N. attenuata*, GLVs stimulate *M. sexta* feeding, and silencing NaSIPK and NaWIPK impairs GLV emission and results in similar larval growth to those fed on wild-type plants, despite their decreased contents of direct defensive compounds(Meldau *et al.*, 2009). This might also account for the normal growth of *M. sexta* on NaMEK2-VIGS plants. Whether NaMKK1 also controls GLV emission requires further investigation.

We identified three more MAPKKs, NaMEK1, NaSIPKK and NaNPK2, which are not involved in the activation of NaSIPK and NaWIPK, but are necessary for TPI activity (Heinrich *et al*., 2011b). Although NaMEK1 and NaSIPKK are important for the accumulation of the JA precursor 12-oxo-phytodienoic acid (OPDA), none of the three MAPKKs can decrease *M. sexta* growth on *N. attenuata* plants silenced in these MAPKKs. In future analysis it would be exciting to identify more MAPKKs, with the help of the complete

genome information and to study the function of other MAPKKs in plant defense against herbivores. The next step after the complete discovery of MAPKKs would be to find their regulators, MAPKKKs, and to unravel the complete molecular signaling pathways leading to the activation of MAPKs. This will provide important insight into the complex signaling structures underlying plant responses after insect's first bite.

CDPKs in *N. attenuata*'s development and hormone regulation

CDPKs have been shown to be involved in development and stress responses (Cheng *et al.*, 2002; Ludwig *et al.*, 2004). Activation of certain CDPKs, like MAPKs, is triggered by biotic and abiotic stresses. For example, two Arabidopsis CDPKs, *CPK3* and *CPK6*, have been shown to be involved in abscisic acid regulation of Ca^{2+} channels in stomatal closure (Mori *et al.*, 2006). *CPK3* and *CPK13* are involved in the defense against the herbivore *Spodoptera littoralis* in Arabidopsis (Kanchiswamy *et al.*, 2010). *CDPK2* in *Nicotiana tabacum* mediates JA and ethylene biosynthesis and also biotic stress responses (Romeis, 2001; Ludwig *et al.*, 2005). Expression analyses revealed that *CDPK4* is likely involved in plant stress reactions (Zhang *et al.*, 2005). In *N. attenuata* four CDPKs, *CDPK2*, *4*, *5* and *8* are transcriptional regulated by herbivory (Wu *et al.*, 2007). A stably silenced *N. attenuata* CDPK line, irCDPK4/5, has highly elevated defense against *Manduca sexta* attack (Yang *et al*., in review), since irCDPK4/5 accumulate high levels of JA and in turn defensive secondary metabolites (Yang *et al*., in review). Additionally, the silenced plants were smaller, with shorter stems, and they have dark green and curly leaves. irCDPK4/5 plants abort most of the flower buds and flowers (Yang *et al*., in review).

In my thesis, I examined the function of CDPK4/5 in stem elongation of *N. attenuata*. Our data indicated that CDPK4/5 negatively regulates JA accumulation in stem during stem elongation. I demonstrate that the highly elevated JA contents in stem account for repression of an important gibberellic acid biosynthesis gene, GA20-oxidase, which is required for plant growth regulation. In *N. tabacum* a CDPK, NtCDPK1 regulates the transcription factor REPRESSION OF SHOOT GROWTH (RSG), which has been suggested to play a role in GA feedback mechanism by regulating biosynthetic enzymes (Ishida *et al.*, 2004; Ishida *et al.*, 2008). Overexpression of NtCDPK1 inhibits the feedback regulation of especially *GA20ox* gene and resulted in the sensitization to a GA biosynthetic inhibitor (Ishida *et al.*, 2008). The authors suggested that NtCDPK1 decodes the Ca^{2+} signal produced by GAs and that it might be responsible for the intracellular localization of RGS (Ishida *et al.*, 2008). Overexpression of *NtCDPK1* inhibited the upregulation of the *GA20ox* in response to a decrease in GA levels

(Ishida *et al.*, 2008). Therefore, different CDPKs appear to be involved in the GA homeostasis by affecting *GA20ox* gene expression as well as other GA biosynthetic genes. Given that DELLA proteins have a feedback function on GA biosynthesis, another scenario for the regulation of *GA20ox* in irCDPK4/5 might be changes in DELLA protein activity, which is resulted from the high JA contents.

DELLA proteins are repressors of GA responses, whose degradation is induced by bioactive GAs (Sun & Gubler, 2004). The fastest documented GA-induced decrease in a DELLA protein occurred 5 to 10 min after GA treatment (Gubler *et al.*, 2002). In *N. attenuata* DELLA proteins have not yet been identified so far. It is interesting to further characterize the functions of DELLA proteins in *N. attenuata*, especially in response to herbivore or pathogen attack and whether they have different roles in growth and development would also be interesting to explore. It has already been shown that DELLAs are important in the response to pathogens by modulating the cross-talk between SA and JA in Arabidopsis (Navarro *et al.*, 2008). Another focus in future work could be the circadian regulation of DELLA, GA biosynthetic genes, and herbivore defense. In Arabidopsis one third of the genes are regulated in a circadian pattern (Pruneda-Paz & Kay, 2010). This enables plants to adapt to changing environmental conditions and daily rhythms of e.g. light and temperature. It has been proposed that hormones might be involved in the relay mechanism to modulate the amplitude and the phase of the output rhythms (Nozue *et al.*, 2007; Michael *et al.*, 2008). It has been shown that the circadian clock gates GA signaling through transcriptional regulation of GA receptors, resulting in higher stability of DELLA proteins during daytime and higher GA sensitivity at night (Arana *et al.*, 2011). The constitutive expression of a GA receptor expands the daily growth period in seedlings of Arabidopsis, and complete loss of DELLA function causes continuous, arrhythmic hypocotyls growth, suggesting that oscillation of GA signaling seems to be critical for rhythmic growth (Arana *et al.*, 2011). It will be interesting to analyze if different time points of GA complementation would yield in different growth phenotype recovery of irCDPK4/5 plants. Microarray experiments of *N. attenuata* plants harvested at different time points could reveal the presumed circadian oscillation of GA receptors and GA biosynthetic genes. Even more importantly, it is interesting to analyze how JA accumulation or JA responses interact with GA homeostasis and signaling after plants are challenged by herbivore attack. Different herbivores may attack at different time periods during day and night, and it has already been shown, that *N. attenuata* plants can even change their time of flowering after being attacked by *M. sexta* larvae, to rather attract hummingbirds for pollination than *M. sexta* moths (Kessler *et al.*, 2010). Normally, *N. attenuata* flowers open at

night and release benzyl acetone (BA), to attract night-active hawkmoth pollinators, which are herbivores and pollinators (Euler & Baldwin, 1996; Kessler & Baldwin, 2007). When *N. attenuata* is attacked by *Manduca* species, it produces flowers with less BA emissions which open in the morning and are preferentially pollinated by day-active hummingbirds (Kessler *et al.*, 2010). This change needs JA signaling (Kessler *et al.*, 2010). We hypothesize that GA signaling, in addition to JA signaling, might also be required for this special response of changing flowering time as well as other responses to environmental stimuli.

Signaling network in *N. attenuata* depends on both CDPKs and MAPKs

In *N. attenuata*, SIPK and WIPK are rapidly activated by herbivore feeding (Wu *et al.*, 2007). It is also conceivable to speculate that both CDPK4 and CDPK5 are activated by herbivory and CDPKs and MAPKs may share certain upstream regulatory elements that dictate synergistic activation of CDPKs and MAPKs in a stimulus-specific manner. Certain stress stimuli could be perceived by a specific receptor/sensor, and thereby activate a set of common signaling elements, which later activate at least two groups of signaling pathways, MAPK and CDPK signaling pathway, resulting in proper downstream reactions, such as gene expression and protein modifications. A model involving both CDPK and MAPK signaling has been proposed for plant innate immune signaling (Boudsocq *et al.*, 2010). Innate immunity represents the first line of inducible defense against microbial infection in plants and animals (Nürnberger *et al.*, 2004; Akira *et al.*, 2006; Boller & Felix, 2009). In both plants and animals recognition of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs, respectively), such as flagellin, induces signaling pathways including MAPK cascades and transcriptional changes to elevate immunity (Asai *et al.*, 2002; Nürnberger *et al.*, 2004; Akira *et al.*, 2006; Boller & Felix, 2009). CDPKs are involved in reprogramming of transcription in plant innate immunity as well (Boudsocq *et al.*, 2010). It has been suggested that CDPKs play a key role in initial MAMP signaling in Arabidopsis by regulating oxidative burst and gene activation induced by the pathogen-derived peptide flagellin flg22 and CDPKs have different functions from MAPKs (Boudsocq *et al.*, 2010). Some genes involved in pathogen defense responses show similar pattern of regulation after flg22 treatment in CDPK- and MAPK- deficient mutants, and regulation of some genes require both CDPK and MAPK pathways (Boudsocq *et al.*, 2010). Using *N. attenuata* lines silenced in MAPKs and CDPKs, it merits further attention to compare the common and specific regulatory targets of these proteins on a genome-wide transcriptional level. Genetic biochemical and analytical chemistry approaches will also help to elucidate the complex interaction between MAPKs and

CDPKs (in Figure 2, we show a simplified working model of current understanding of the interaction between MAPK and CDPK signaling cascades after herbivore attack in *N. attenuata*).

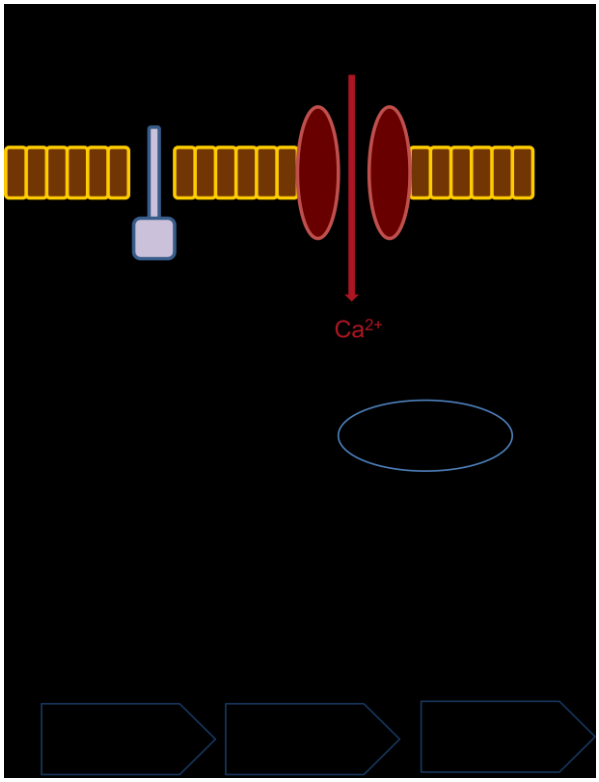


Figure 2. Hypothetical signaling network of MAPK and CDPK cascades after herbivore attack.

CDPK and MAPK cascades might interact after herbivore attack on gene regulation. *TF*, transcription factors. This model was adapted from Boudsocq *et al*., 2010.

GA and JA interaction supports the Optimal Defense Theory

N. attenuata is challenged by various environmental stresses and responses to these stresses requires energy and resources. For example, to cope with herbivore attack, plants produce defensive compounds, whose biosynthesis is usually energy- and nutrient-demanding (Karban & Baldwin, 1997). This balance of resource allocation between fitness and defense is usually explained by the Optimal Defense Theory (Mckey, 1974; Boege & Marquis, 2005), namely, if defenses are both costly and beneficial, natural selection will shape the growth and defense regulation in a manner that produces best benefit-cost ratios. It has been speculated that in a certain plant species, the most important tissues/organs for fitness should be protected the most and these highly protected tissue types change over plants' development. The irCDPK4/5 line is strongly impaired in its fitness, as the stem grows shorter, the apical dominance is decreased, and flowers have low fertility and produce little seeds. On the other hand it has highly elevated levels of JA and a large increase in all known defensive compounds, such as nicotine, phenolic compounds, and TPIs. This high JA level causes low GA biosynthesis by downregulation of the *GA20ox* gene expression. By contrast, JA-deficient

plants or plants impaired in JA signaling, like irAOC and irCOI plants, have increased stem growth compared to WT plants (manuscript 3). The exact mechanism by which JA affect transcript accumulation of GA biosynthetic genes as well as GA receptor genes is still not known. It is very likely that GA and JA are both involved in *N. attenuata* growth, fitness, and defense responses, and they are both important for optimal defense. Another study demonstrated that another phytohormone, ethylene, is involved in the optimal defense and fitness of *N. attenuata* (Diezel *et al.*, 2011). Herbivory-elicited ethylene production changed dramatically in different developmental stages and a similar pattern was found for JA and its active form, JA-Ile (Diezel *et al.*, 2011). Interestingly, the degrading inducibility of JA and ethylene was almost completely recovered after flower removal by decapitation to inhibit floral signals (Diezel *et al.*, 2011). It is known that GA promotes flowering in Arabidopsis (Blazquez *et al.*, 1998) and gibberellin is also necessary for xylem expansion (Ragni *et al.*, 2011). Overexpression of a JA biosynthesis gene, *DGL*, in Arabidopsis results in higher JA levels and loss of apical dominance, flowers are fertile but have shorter anthers which results in pollination failure (Hyun *et al.*, 2008). High JA levels in *dgl-D* mutant might decrease GA contents or activity, which is necessary for flower development (Cheng *et al.*, 2004). It is likely that find-tuned balance between JA and GA signaling is one of the key elements in plant optimal fitness and defense.

Summary

Protein kinases modulate developmental and adaptation processes in the plant kingdom. While MAPK signaling has been intensively studied for the involvement in growth, defense and growth regulation, more and more studies have shown that CDPKs also function in these physiological processes. *N. attenuata* is an annual solanaceous plant, which germinates only after fire. In its natural habitat in the Great Basin Desert in Utah, USA, after being attacked by the specialist, tobacco hornworm *Manduca sexta*, *N. attenuata* has developed many specific and fine-tuned responses to cope with herbivore challenges. It has been shown that two MAPKs, NaSIPK and NaWIPK are involved in defense against *M. sexta* attack, by controlling the plant hormone, jasmonic acid (JA). The burst of JA after herbivore recognition is important to induce defense responses, such as the production of small secondary metabolites (e.g. nicotine) and defensive proteins – trypsin proteinase inhibitor (TPI).

MAPKs are the last element in a MAPK cascade: MAPKKKs activate MAPKKs, which in turn phosphorylate MAPKs. In order to investigate the function of MAPK cascades in plant-herbivore interactions, using *N. attenuata* and *M. sexta* as the model system, I studied the function of five MAPKKs in plant defense against herbivores. A virus-induced gene silencing method was used to transiently knock down the transcript levels of these five MAPKKs. These lines were used to analyze the role of MAPKKs in regulating the accumulation of phytohormones JA and ethylene, the MAPKs, NaSIPK and NaWIPK, and the activity of TPI. *M. sexta* performance assays were used to evaluate the impact of the five MAPKKs on larval growth. It was found that one MAPKK, NaMEK2, plays an important role in activating NaSIPK, and is therefore involved in JA accumulation and ethylene production after herbivory. Although the other four MAPKKs do not affect NaSIPK activity levels, like NaMEK2, they are also involved in the regulation of TPI activity. Moreover, two MAPKKs, NaMEK1 and NaSIPKK, are important for early steps of JA biosynthesis, because they function in the regulation of a JA precursor, 12-oxo-phytodienoic acid (OPDA). However, none of these five MAPKKs was sufficient to have a detectable impact on larval growth of *M. sexta*. This might be due to redundancy of these MAPKKs in regulating herbivore resistance.

After wounding or herbivory, highly elevated JA levels were found in irCDPK4/5 plants, which were silenced in two CDPKs, CDPK4 and CDPK5. irCDPK4/5 also exhibited

stunted stem elongation. Using irCDPK4/5 as the model, I sought to unravel the function of JA in *N. attenuata*'s growth. I found remarkably high levels of JA in irCDPK4/5 stem, and genetic analysis using plants deficient in JA accumulation or signaling, which were crossed with irCDPK4/5, indicated that high JA levels accounted for the impaired stem growth. Applying another phytohormone, gibberellic acid (GA), to irCDPK4/5 largely recovered its stem growth to almost wild-type level. Using quantitative real time PCR, it was shown, that high JA levels repress an important GA biosynthesis gene, GA20-oxidase (*GA20ox*). Using virus-induced gene silencing, I knocked down *GA20ox* transcripts and further confirmed that GA20ox is important for stem growth. This work revealed a novel interaction between JA and GA: JA antagonizes the biosynthesis of GAs by suppressing the transcript levels of *GA20ox*.

Zusammenfassung

Die Anpassung von Pflanzen an ihre Umwelt wird zu einem großen Teil von Protein Kinasen reguliert. Pflanzen können durch diese Kinasen ihre Entwicklung und ihre Verteidigung in Antwort auf biotische und abiotische Einflüsse steuern. Während mitogen-aktivierte Protein Kinasen (MAPKs) schon sehr intensiv im Zusammenhang mit Wachstum, Verteidigung und Wachstumsregulation untersucht wurden, wird jetzt erst nach und nach gezeigt, dass auch calcium-aktivierte Protein Kinasen (CDPKs) in diesen Prozessen eine wichtige Rolle spielen. Die wilde Tabakpflanze *Nicotiana attenuata* gehört zu den Solanaceaen und keimt speziell nach Bränden. Natürlicherweise kommt sie in der Great Basin Wüste in Utah, USA vor. Da sie zu den ersten Pflanzen nach einem Brand zählt, wird sie besonders häufig von Fraβfeinden bedroht. Um sich anzupassen, hat sie spezielle und komplex regulierte Mechanismen entwickelt. Die Abwehr der Tabakpflanze gegen den Tabakhornwurm *Manduca sexta* wurde bereits sehr intensiv studiert. Es wurde gezeigt, dass zwei MAPKs, NaSIPK und NaWIPK in Verteidigungsantworten gegen *M. sexta* Attacken involviert sind, in dem sie ein Pflanzenhormon, Jasmonsäure (JA) aktivieren. Der Anstieg von JA nach der Erkennung von Herbivoren ist ein wichtiger Faktor, der Verteidigungsantworten wie zum Beispiel die Produktion von Sekundärmetaboliten (z.B. Nikotin) und von Verdauungshemmern (z.B. Trypsin Proteinase Inhibitoren, TPI) initiiert.

MAPKs sind die letzten Regulationselemente einer Signalkaskade. Diese beginnt mit der Aktivierung von einer oder mehreren MAPKKKs, diese phosphorylieren dann MAPKKs, welche dann MAPKs aktivieren. Um die Funktion dieser Signalkaskaden in der besser zu verstehen, habe ich fünf MAPKKs und ihre Funktion in der Verteidigung gegen *M. sexta* erforscht. Für meine Versuche habe ich die RNA Transkripte der MAPKKs mit Hilfe einer Virus-basierten Methode herunter reguliert, um so die MAPKKs auszuschalten. Die dadurch veränderten Pflanzen habe ich dann auf die Aktivierung der MAPKs, NaSIPK und NaWIPK, die Phytohormone JA und Ethylen, sowie die Veränderungen der TPI Aktivität untersucht. Die MAPKKs, NaMEK1 und NaSIPKK spielen eine Rolle im frühen JA Signalweg, da sie die Akkumulation eines JA Vorproduktes, 12-oxo-phytodien Säure, OPDA, beeinflussen. Ich fand heraus, dass eine MAPKK, NaMEK2 einen großen Einfluss auf die Aktivität von NaSIPK hat und dadurch in die Akkumulation von JA und Ethylen nach Herbivorbefall involviert ist. Alle fünf MAPKKs sind wichtig für die TPI Regulation. Um den Einfluss der MAPKKs auf das Raupenwachstum zu beleuchten, führte ich Raupenassays mit *M. sexta*

Larven durch. Das Ausschalten einzelner MAPKK war nicht ausreichend, um das Wachstum von *M. sexta* Larven zu behindern.

Nach Verwundung oder Herbivorie wurden in irCDPK4/5 Pflanzen, welche in den CDPKs CDPK4 und CDPK5 herunter reguliert waren, sehr hohe JA Mengen gemessen. Diese Pflanzen zeigten auch ein verkümmertes Stammwachstum. Daher habe ich sie als Modelpflanzen gewählt, um den Einfluss von JA auf das Stammwachstum von *N. attenuata* zu untersuchen. In den Stämmen von irCDPK4/5 Pflanzen war die basale JA Menge sehr hoch, und durch die genetische Analyse durch die Kreuzung mit transgenen Pflanzen, mit vermindertem JA-Signalwegen oder JA-Akkumulationen, konnte ich zeigen, dass wahrscheinlich JA für den verringerten Stammwuchs verantwortlich ist. Durch die Behandlung der Pflanzen mit einem anderen Pflanzenhormon, Gibberellinsäure (GA), erholte sich der Stamm und erreichte fast die gleiche Höhe wie Wildtyppflanzen. Mittels quantitativer Echtzeit-PCR wurde gezeigt, dass ein wichtiges GA Biosynthese Gen, GA20-Oxidase (GA20ox) in irCDPK4/5 Pflanzen unterdrückt wird. Die Expression dieses Gens inhibierte ich mit Hilfe der bereits erwähnten Virus-basierten Genausschaltung in unveränderten Pflanzen, wodurch diese im Laufe ihrer Entwicklung dann ebenfalls verringertes Stammwachstum zeigten. Diese Arbeit zeigt eine neue Interaktion zwischen JA und GA: JA verhindert die Biosynthese von GA durch die Unterdrückung der GA20ox Transkription.

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Curriculum vitae

Personal Information

Name: Maria Heinrich

Date of birth: 26. 07. 1984

Place of birth: Kiew, Ukraine

Nationality: German

Current address: Über dem Dorfe 55, 07745 Jena

E-mail: mheinrich@ice.mpg.de

Research Experience

Since 01/2009	PhD Thesis at the Max-Planck-Institute for Chemical Ecology Dissertation on: "*Nicotiana attenuata* protein kinases mediate plant growth and resistance to insect herbivores" Main focus on: Molecular Biology (DNA-cloning, PCR, transformation of plants and bacteria, qRT-PCR) Protein Biochemistry (gel-electrophoresis, immunodetection, proteomics) Analytical Chemistry (HPLC, LC-MS) Ecology (field studies, herbivore assays)
01/2008- 12/2008	Diploma thesis at the Institute of General Botany, FSU Jena „Charakterisierung des pflanzenähnlichen Cryptochroms in *Chlamydomonas reinhardtii*" Main focus on: Molecular Biology (DNA- cloning, PCR, sequence analysis) Protein Biochemistry (gel-electrophoresis; immunodetection; protein- overexpression for antibody production) *E. coli* culturing (recombinant protein expression, extraction and purification)
09/2007- 12/2007	Student Assistant at the Institute of General Botany, FSU Jena
05/2007	Internship in Moscow, Lomonosov- University, Biology Department, Main focus on: Chemotaxis in the green algae *Chlamydomonas reinhardtii*

Education

10/2003- 12/2008	Diploma in Biology at the Friedrich- Schiller- University Jena Final Grade: 2.0
08/1994- 06/2003	Abitur Ernst- Abbe- Gymnasium Jena (1.6)

Publications

- Heinrich, M., Baldwin, I. T., Wu, J. (2011). Two mitogen-activated protein kinase kinases, MKK1 and MEK2, are involved in wounding- and specialist lepidopteran herbivore *Manduca sexta*-induced responses in *Nicotiana attenuata*. Journal of Experimental Botany, 62, 4355-4365.
- Heinrich, M., Baldwin, I. T., Wu, J. (2011). Three MAPK kinases, MEK1, SIPKK and NPK2, are not involved in activation of SIPK after wounding and herbivore feeding but important for accumulation of trypsin proteinase inhibitors. Plant Molecular Biology Reporter. doi:10.1007/s11105-011-0388-0.
- Bozorov, T. A., Pandey, S. P., Dinh, T. S., Kim, S.-G., Heinrich, M., Gase, K., Baldwin, I. T. (in press). Dicer-like proteins and their role in plant-herbivore interactions in *Nicotiana attenuata*. Journal of Integrative Plant Biology. doi:10.1111/j.17447909.2012.01104.
- Heinrich, M., Hettenhausen, C., Lange, T., Wünsche, H., Fang, J., Baldwin, I.T., Wu, J. (2012). Jasmonic acid specifically suppresses the transcript accumulation of *GA20ox* gene and thus inhibits the biosynthesis of gibberellic acid in the stem of irCDPK4/5 plants (will be submitted to PLOS Biology).

Oral presentations

- Heinrich M. NaMKK1 regulates *N. attenuata´*s sensitivity to allelopathic cues. 9th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Feb 2010
- Heinrich M. Discovery of gibberellin actions in a post-fire wild tobacco plant. Seminar, TU Braunschweig, Institut für Pflanzenbiologie, Braunschweig, DE, Dec 2011

Poster presentations

- Heinrich M., Baldwin I.T., Wu J. A calcium sensing protein, CDPK5, controls herbivory-induced jasmonic acid accumulation and plant development in *N. attenuata*. 14th Symposium on Insect-Plant Interactions, Wageningen, NL, Aug 2011
- Heinrich M. NaCDPK5-a central regulator of primary and secondary metabolism in *N. attenuata*. 10th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Feb 2011
- Heinrich M. MAPKKs: teamwork in plant defense. 5th Plant Science Student Conference, Halle (Saale), DE, Jun 2009
- Heinrich M. MAPKKs: teamwork in plant defense. 8th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Mar 2009

2011 IMPRS **travel award** for the best Poster, 10th IMPRS Symposium

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Maria Heinrich

Jena,

Selbstständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, dass ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

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Maria Heinrich

Jena,